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CONTENTS

NUMBER 1, JANUARY, 1949

Studies in Diabetic Acidosis and Coma, with Particular Emphasis on the Retention of Administered Potassium. T. S. DANOWSKI, J. H. PETERS, J. C. RATHBUN, J. M. QUASHNOCK, AND L. GREENMAN.	1
Simultaneous Measurement of Effective Renal Blood Flow and Cardiac Output in Resting Normal Subjects and Patients with Essential Hypertension. ALFRED A. BOLOMEY, ALEXANDER J. MICHEL, CATHARINE MICHEL, ERNEST S. BREED, GEORGE E. SCHREINER, AND HENRY D. LAUSON.	10
Use of Blood Donors with Positive Serologic Tests for Syphilis—with a Note on the Disappearance of Passively Transferred Reagin. MARK M. RAVITCH, THOMAS W. FARMER, AND BARBARA DAVIS.	18
Human Prothrombin: Quantitative Studies on the Plasma Labile Factor and the Restorative Effects of Normal, Hypofibrinogenemic, and Hemophilic Plasma on the Prothrombin of Stored Plasma. BENJAMIN ALEXANDER AND ANDRE DE VRIES.	24
A Study of the Rate of Disappearance of a Digitalis Glycoside (Lanatoside C.) from the Blood of Man. MEYER FRIEDMAN AND RENÉ BINE, JR.	32
The Renal Regulation of Acid-Base Balance in Man. III. The Reabsorption and Excretion of Bicarbonate. R. F. PITTS, J. L. AYER, AND W. A. SCHIESS.	35
The Bioassay of Adrenal Corticoids in the Urine of Patients with Congestive Heart Failure. ALVIN E. PARRISH.	45
Autoantibodies in Human Glomerulonephritis. KURT LANGE, MICHAEL M. A. GOLD, DAVID WEINER, AND VERA SIMON.	50
The Hyaluronic Acid of Synovial Fluid in Rheumatoid Arthritis. CHARLES RAGAN AND KARL MEYER.	56
Blood Oxygen Studies in Patients with Polycythemia and in Normal Subjects. L. R. WASSERMAN, R. L. DOBSON, AND J. H. LAWRENCE.	60
The Phosphorus Turnover of Carcinoma of the Human Stomach as Measured with Radioactive Phosphorus. JOHN SCHULMAN, JR., MARLENE FALKENHEIM, AND SEYMOUR J. GRAY	66
Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXVII. The Metal-Combining Globulin of Human Plasma. DOUGLAS M. SURGENOR, BERNARD A. KOECHLIN, AND LAURENCE E. STRONG.	73
Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. CHARLES E. RATH AND CLEMENT A. FINCH	79
Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXIX. The Anemia of Infection. Studies on the Iron-Binding Capacity of Serum. G. E. CARTWRIGHT AND M. M. WINTROBE.	86
Potassium Deficiency and the Role of the Kidney in its Production. ROBERT TARAIL AND J. RUSSELL ELKINTON.	99
Can Vascular Shunting Be Induced in the Kidney by Vasoactive Drugs? FRANÇOIS C. REUBI AND HENRY A. SCHROEDER.	114
Electrophrenic Respiration. II. Its Use in Man. JAMES L. WHITTENBERGER, STANLEY J. SARNOFF, AND ESTHER HARDENBERGH.	124
The Volume of the Lung Determined by Helium Dilution. Description of the Method and Comparison with Other Procedures. GEORGE R. MENEELY AND NOLAN L. KALTREIDER	129
Studies on Human Alcaptonuria. Effect of Thiouracil, Para-Aminobenzoic Acid and Di-Iodotyrosine on Excretion of Homogentisic Acid. ABRAHAM G. WHITE, JULIUS G. PARKER, AND FRANK BLOCK.	140

The Significance of the Difference in Systemic Arterial and Venous Plasma Concentrations in Renal Clearance Methods. CLAUS BRUN, TAGE HILDEN, AND FLEMMING RAASCHOU....	144
Studies on Pain: Measurements of Pain Intensity in Childbirth. JAMES D. HARDY AND CARL T. JAVERT.....	153
Methods for Measuring the Activity of Components of the Streptococcal Fibrinolytic System, and Streptococcal Desoxyribonuclease. L. R. CHRISTENSEN.....	163
The Effect in Patients of Streptococcal Fibrinolysin (Streptokinase) and Streptococcal Desoxyribonuclease on Fibrinous, Purulent, and Sanguinous Pleural Exudations. WILLIAM S. TILLET AND SOL SHERRY.....	173

NUMBER 2, MARCH, 1949

The Behavior of Radioiodine in the Blood. WILLIAM M. MCCONAHEY, F. RAYMOND KEATING, JR., AND MARSCHELLE H. POWER.....	191
Induced Insulin Resistance in the Rabbit. FRANCIS C. LOWELL AND WILLIAM FRANKLIN...	199
Relative Measurement <i>In Vivo</i> of Accumulation of Radioiodine by the Human Thyroid Gland: Comparison with Radioactivity in Peripheral Tissues. THOMAS J. LUELLEN, F. RAYMOND KEATING, JR., MARVIN M. D. WILLIAMS, JOSEPH BERKSON, MARSCHELLE H. POWER, AND WILLIAM M. MCCONAHEY.....	207
The Measurement of the Iodine-Accumulating Function of the Human Thyroid Gland. F. RAYMOND KEATING, JR., J. C. WANG, THOMAS J. LUELLEN, MARVIN M. D. WILLIAMS, MARSCHELLE H. POWER, AND WILLIAM M. MCCONAHEY.....	217
The Validity of the Electrokymographic Method for Measurement of Diameter Change of the Aorta and Pulmonary Artery during Circulatory Disturbance. CALVIN F. KAY, JAMES W. WOODS, JR., HARRY F. ZINSSER, JR., AND J. MALVERN BENJAMIN, JR.....	228
Studies of the Role of the Liver in Human Carbohydrate Metabolism by the Venous Catheter Technic. I. Normal Subjects under Fasting Conditions and following the Injection of Glucose. PHILIP K. BONDY, DAVID F. JAMES, AND BETTY W. FARRAR.....	238
Metabolic Studies on Protein-Depleted Patients Receiving a Large Part of their Nitrogen Intake from Human Serum Albumin Administered Intravenously. CHRISTINE WATERHOUSE, SAMUEL H. BASSETT, AND JACOB W. HOLLER.....	245
Congenital Methemoglobinemia. A Clinical and Biochemical Study of a Case. HOWARD A. EDER, CLEMENT FINCH, AND RALPH W. MCKEE.....	265
The Effects of Induced Malaria, Acute Starvation and Semi-Starvation on the Electrophoretic Diagram of the Serum Proteins of Normal Young Men. HENRY LONGSTREET TAYLOR, OLAF MICKELSEN, AND ANCEL KEYS.....	273
Complement and its Components in Human Cerebrospinal Fluid. MICHAEL HEIDELBERGER AND ROBERT H. MULLER.....	282
Hematologic Observations in Patients with Chronic Hepatic Insufficiency. THOMAS JARROLD AND RICHARD W. VILTER.....	286
The Cardiovascular Effects of Acutely Induced Hypothermia. O. PREC, R. ROSENMAN, K. BRAUN, S. RODBARD, AND L. N. KATZ.....	293
The Circulatory Responses to Hyperthermia Induced by Radiant Heat. O. PREC, R. ROSENMAN, K. BRAUN, R. HARRIS, S. RODBARD, AND L. N. KATZ.....	301
The Relation of Influenza Virus and Bacteria in the Etiology of Pneumonia. ELIZABETH STARBUCK MAXWELL, THOMAS G. WARD, AND THOMAS E. VAN METRE, JR.....	307
Determination of the Plasma Glycine after Gelatin Feeding as a Diagnostic Procedure for Pancreatic Fibrosis. HALVOR N. CHRISTENSEN AND HARRY SHWACHMAN.....	319
Studies in Cirrhosis of the Liver. I. Relationship between Plasma Volume, Plasma Protein Concentrations and Total Circulating Proteins. GLENN I. HILLER, ELSTON R. HUFFMAN, AND STANLEY LEVEY.....	322

The Agglutination of Autoclaved Hemolytic Streptococci by Serum from Patients with Rheumatic Fever and Other Conditions. SUNG J. LIAO.....	331
The Clotting Behavior of Human "Platelet-Free" Plasma: Evidence for the Existence of a "Plasma Thromboplastin." C. LOCKARD CONLEY, ROBERT C. HARTMANN, AND WILLIAM I. MORSE, II.	340
The Hemodynamic Effects of Hypotensive Drugs in Man. I. Veratrum Viride. EDWARD D. FREIS, JOSEPH R. STANTON, JAMES W. CULBERTSON, JULIUS LITTER, MEYER H. HALPERIN, CHARLES H. BURNETT, AND ROBERT W. WILKINS.....	353
Some Observations on the Ballistocardiographic Pattern, with Special Reference to the H and K Waves. JOHN L. NICKERSON.....	369
Transfers of Potassium in Renal Insufficiency. J. RUSSELL ELKINTON, ROBERT TARAIL, AND JOHN P. PETERS.....	378
Complement and its Component Fractions in Cerebrospinal Fluid in Inflammatory Cerebrospinal Diseases. SOPHIE SPICER, EMANUEL APPELBAUM, AND DAVID D. RUTSTEIN.....	389
The Effect of Adrenocorticotrophic Hormone in Congenital Adrenal Hyperplasia with Virilism and in Cushing's Syndrome Treated with Methyl Testosterone. ROGER A. LEWIS AND LAWSON WILKINS.....	394
A Brief Sketch of the History of the American Society for Clinical Investigation. J. HAROLD AUSTIN.....	401

NUMBER 3, MAY, 1949

Some Observations on the Development of Hypokaliemia during Therapy of Diabetic Acidosis in Juvenile and Young Adult Subjects. L. GREENMAN, F. M. MATEER, R. C. GOW, J. H. PETERS, AND T. S. DANOWSKI.....	409
The Effect of Bilateral Stellate Ganglion Block on the Cerebral Circulation in Normotensive and Hypertensive Patients. M. H. HARMEL, J. H. HAFKENSCHIEL, G. M. AUSTIN, C. W. CRUMPTON, AND S. S. KETY.....	415
Peptidase Activity in Human Serum Following Bone Fracture. KARL STERN, ANDREA M. CULLEN, AND VIRGINIA T. BARBER.....	419
The Renal Regulation of Acid-Base Balance in Man. IV. The Nature of the Renal Compensations in Ammonium Chloride Acidosis. O. W. SARTORIUS, J. C. ROEMMELT, AND R. F. PITTS.....	423
The Effects of Histamine on Renal Function in Hypertensive and Normotensive Subjects. F. C. REUBI AND P. H. FUTCHER.....	440
Studies of Coproporphyrin. I. The Per Diem Excretion and Isomer Distribution of Coproporphyrin in Normal Human Urine. CECIL JAMES WATSON, VIOLET HAWKINSON, SAMUEL SCHWARTZ, AND DONALD SUTHERLAND.....	447
Studies of Coproporphyrin. II. An Investigation of the Contribution to the Urinary Coproporphyrin of Hemoglobin and of Bacterial Metabolism in the Intestinal Tract. EVREL A. LARSON AND CECIL JAMES WATSON.....	452
Studies of Coproporphyrin. III. Idiopathic Coproporphyrinuria; a Hitherto Unrecognized Form Characterized by Lack of Symptoms in Spite of the Excretion of Large Amounts of Coproporphyrin. CECIL JAMES WATSON, SAMUEL SCHWARTZ, WILLIAM SCHULZE, LEON O. JACOBSON, AND RALPH ZAGARIA.....	465
Intra-Articular Temperature as a Measure of Joint Reaction. STEVEN M. HORVATH AND JOSEPH L. HOLLANDER.....	469
III. The Fate of Citrate in Erythroblastotic Infants Treated with Exchange Transfusion. IRVING B. WEXLER, JOSEPH B. PINCUS, SAMUEL NATELSON, AND JULIUS K. LUGOVOY...	474
Electrolyte Changes in Human Striated Muscle in Acidosis and Alkalosis. GILBERT H. MUDGE AND KATHERINE VISLOCKY.....	482

Studies on Free Erythrocyte Protoporphyrin, Serum Iron, Serum Iron-Binding Capacity and Plasma Copper During Normal Pregnancy. JANE FAY, G. E. CARTWRIGHT, AND M. M. WINTROBE.....	487
Studies on the Activation of a Serum "Prothrombin-Converting Factor." RALPH F. JACOX	492
Studies in Iron Transportation and Metabolism. VII. Evidence Obtained with Radioactive Iron that "Easily Split-Off" Blood Iron is an Artefact. MOISES GRINSTEIN AND CARL V. MOORE.....	505
A Comparison of the Direct Fick and the Grollman Methods for Determination of the Cardiac Output in Man. LARS WERKÖ, STIG BERSEUS, AND HENRIK LAGERLÖF.....	516
A Factor Determining the Location of Pseudofractures in Osteomalacia. M. LE MAY AND J. W. BLUNT, JR.....	521
The Oxygen Consumption of the Normal and the Diseased Human Kidney. WALTER H. CARGILL AND JOHN B. HICKAM.....	526
The Measurement of Glomerular and Tubular Plasma Flow in the Normal and Diseased Human Kidney. WALTER H. CARGILL.....	533
Hypervolemic Anemia in Cirrhosis. JEANNE C. BATEMAN, HAROLD M. SHORR, AND TORBJORN ELGVIN.....	539
The Effect of Hyaluronidase on the Absorption of Parenterally Administered Radioactive Plasma Proteins in the Dog. HENRY H. BANKS, ARNOLD M. SELIGMAN, AND JACOB FINE	548
The Acceleration of Linear Flow in the Deep Veins of the Lower Extremity of Man by Local Compression. JOSEPH R. STANTON, EDWARD D. FREIS, AND ROBERT W. WILKINS.....	553
The Effect of Exercise and Body Position on the Venous Pressure at the Ankle in Patients Having Venous Valvular Defects. ALBERT A. POLLACK, BOWEN E. TAYLOR, THOMAS T. MYERS, AND EARL H. WOOD.....	559
The Inhibition of Frostbite Wheals by the Iontophoresis of Antihistaminic Agents. MARTIN B. MACHT, MORTIMER E. BADER, AND JERE MEAD.....	564
The Construction of Normal Standards for Cardiac Output in Man. J. M. TANNER.....	567

NUMBER 4, JULY, 1949

An Evaluation of Human Serum Albumin in the Treatment of Cirrhosis of the Liver. WILLIAM W. FALON, RICHARD D. ECKHARDT, T. LYNCH MURPHY, ARNOLD M. COOPER, AND CHARLES S. DAVIDSON.....	583
The Effect of Human Serum Albumin, Mercurial Diuretics, and a Low Sodium Diet on Sodium Excretion in Patients with Cirrhosis of the Liver. WILLIAM W. FALON, RICHARD D. ECKHARDT, ARNOLD M. COOPER, AND CHARLES S. DAVIDSON.....	595
Improvement of Active Liver Cirrhosis in Patients Maintained with Amino Acids Intravenously as the Source of Protein and Lipotropic Substances. RICHARD D. ECKHARDT, WILLIAM W. FALON, AND CHARLES S. DAVIDSON.....	603
The Effects of Dihydroergocornine on the Circulation in the Extremities of Man. DANIEL W. HAYES, KHALIL G. WAKIM, BAYARD T. HORTON, AND GUSTAVUS A. PETERS.....	615
Studies of Coproporphyrin. IV. The Per Diem Excretion and Isomer Distribution in the Urine in Infectious Hepatitis, Infectious Mononucleosis, and Mechanical Jaundice. CECIL JAMES WATSON, VIOLET HAWKINSON, RICHARD B. CAPPS, AND EMANUEL M. RAPPAPORT	621
Studies on Vasomotor Tone. I. The Effect of the Tetraethylammonium Ion on the Peripheral Blood Flow of Normal Subjects. SIBLEY W. HOOBLER, S. DONALD MALTON, H. THOMAS BALLANTINE, JR., SAUL COHEN, ROSALIE B. NELIGH, M. M. PEET, AND RICHARD H. LYONS.....	638
The Effect of Anoxic Anoxia on the Human Kidney. EUGENE Y. BERGER, MORTON GALDSTON, AND SEYMOUR A. HORWITZ.....	648

Determination of Adrenergic and Cholinergic Substances in the Blood of Man. MARC J. MUSSER AND ELIZABETH A. GRIMM.....	653
Concentration-Time Course in the Plasma of Man of Radiomercury Introduced as a Mercurial Diuretic. S. A. THREEFOOT, C. T. RAY, G. E. BURCH, J. A. CROSVICH, J. P. MILNOR, W. OVERMAN, AND W. GORDON.....	661
A Study of Certain Aspects of Blood Coagulation in the Postoperative State in Congestive Heart Failure and in Thrombophlebitis. NOBLE O. FOWLER.....	671
Studies of Lung Volumes and Intrapulmonary Mixing: Notes on Open-Circuit Methods, Including Use of a New Pivoted Type Gasometer for Lung Clearance Studies. J. B. BATEMAN, WALTER M. BOOTHBY, AND H. FREDERIC HELMHOLTZ, JR.....	679
Observations on the Proteolytic Activity <i>In Vitro</i> at Neutral Reaction of Gastric Juice from Patients with Sprue. HERBERT J. FOX.....	687
Sodium and Chloride Depletion in Acute Porphyria with Reference to the Status of Adrenal Cortical Function. F. T. G. PRUNTY.....	690
Treatment of Nephrosis with Concentrated Human Serum Albumin. I. Effects on the Proteins of Body Fluids. JOHN A. LUETSCHER, JR., ALASTAIR D. HALL, AND VIRGINIA L. KREMER.....	700
Mucolytic Enzyme Systems. VIII. Inhibition of Hyaluronidase by Human Blood Serum During the Normal Menstrual Cycle and Pregnancy. ERICK Y. HAKANSON AND DAVID GLICK.....	713
Studies on Amino Acid Excretion in Man. III. Amino Acid Levels in Plasma and Urine of Normal Men Fed Diets of Varying Protein Content. JOSEPH B. KIRSNER, A. LEONARD SHEFFNER, AND WALTER LINCOLN PALMER.....	716
Factors in the Treatment of Laennec's Cirrhosis. I. Clinical and Histological Changes Observed During a Control Period of Bed-Rest, Alcohol Withdrawal, and a Minimal Basic Diet. GERALD KLATSKIN AND RAYMOND YESNER.....	723
Oxygen Saturation of Sternal Marrow Blood in Polycythemia Vera. BERNARD M. SCHWARTZ AND DANIEL STATS.....	736
IV. Response of Citric Acid Levels of Normal Adults and Children to Intramuscular Injection of Epinephrine. JOSEPH B. PINCUS, SAMUEL NATELSON, AND JULIUS K. LUGOVY.....	741
Studies of the Effects of Flavonoids on Roentgen Irradiation Disease. II. Comparison of the Protective Influence of Some Flavonoids and Vitamin C in Dogs. JOHN B. FIELD AND PAUL E. REKERS.....	746
The Plasma Viscosity in Pulmonary Tuberculosis and Rheumatic Diseases. JOHN HOUSTON, R. B. WHITTINGTON, I. C. COWAN, AND JOHN HARKNESS.....	752
Proceedings of the Forty-first Annual Meeting of the American Society for Clinical Investigation, Held in Atlantic City, N. J., May 2, 1949.....	764

NUMBER 5, PART 1, SEPTEMBER, 1949

SYMPOSIUM ON ANTIBIOTICS

Foreword.....	i
The Elimination of Penicillin G in Bilaterally Nephrectomized Dogs. WILLIAM H. ANDERSON AND ROLF BRODERSEN.....	821
Studies on Hypersensitivity to Penicillin. I. Incidence of Reactions in 1303 Patients. MARK H. LEPPER, HARRY F. DOWLING, JAY A. ROBINSON, THOMAS E. STONE, ROBERT L. BRICKHOUSE, ESTON R. CALDWELL, JR., AND RICHARD L. WHELTON.....	826
The Recovery of Bacteria from the Toxic Effects of Penicillin. HARRY EAGLE.....	832
Amperometric Micro-Titration of Streptomycin and Dihydrostreptomycin. JOHN B. CONN AND SARA L. NORMAN.....	837

9

A New Linear Diffusion Method for the Microbiological Assay of Streptomycin and Dihydrostreptomycin. J. M. McGUIRE, W. W. DAVIS, T. V. PARKE, AND W. A. DAILY.....	840
Quantitative Determination of Dihydrostreptomycin by Periodate Oxidation. EDWARD A. GARLOCK, JR., AND DONALD C. GROVE.....	843
Investigations on the Streptomycins. GEOFFREY RAKE, FELIX PANSY, WILLIAM P. JAMBOR, AND RICHARD DONOVICK.....	846
The Effect of Streptomycin Derivatives on Streptomycin-Dependent and -Resistant Strains of Bacteria. WALTER A. WINSTEN.....	850
The Development of Resistance of <i>Salmonella Typhosa</i> to the Fatty Acid Salts of Streptomycin and Dihydrostreptomycin. HENRY WELCH, HENRY FISCHBACH, C. W. PRICE, AND C. H. SHAFFER.....	856
The Effects of Normal Horse Serum on the <i>In Vitro</i> Activity of Tyrothricin. ROBERT J. REEDY AND STANLEY W. WOLFSON.....	861
<i>In Vitro</i> Studies on Possible Synergistic Action Between Penicillin and Bacitracin. MARVIN C. BACHMAN.....	864
Comparison of the Action of Streptomycin, Polymyxin B., Aureomycin and Chloromycetin on <i>H. Pertussis</i> , <i>H. Parapertussis</i> , <i>H. Influenzae</i> and Five Enteric Strains of Gram-Negative Bacilli. HATTIE E. ALEXANDER, GRACE LEIDY, AND WINIFRED REDMAN.....	867
Experimental Studies on the Action of Streptomycin, Aureomycin, and Chloromycetin on <i>Brucella</i> . ELLARD M. YOW AND WESLEY W. SPINK.....	871
Antigenic and Enzyme System Changes in Beta Hemolytic Streptococci Resistant to Penicillin, Streptomycin, Bacitracin and Aureomycin. HORACE M. GEZON AND DORCAS M. FASAN.....	886
Patterns of Bacterial Resistance to Penicillin, Aureomycin, and Streptomycin. M. DEMEREC.....	891
The Partial Purification and Properties of Antibiotic Substances from the Sweet Potato Plant (<i>Ipomoea Batatas</i>). B. H. BRUCKNER, HAZEL H. MCKAY, P. S. SCHAFFER, AND THOMAS D. FONTAINE.....	894
The Partial Purification and Properties of Antibiotic Substances from the Banana (<i>Musa Sapientum</i>). WILLIAM E. SCOTT, HAZEL H. MCKAY, P. S. SCHAFFER, AND THOMAS D. FONTAINE.....	899
Antibiotic Studies on an Extract from <i>Leptotaenia Multifida</i> . G. A. MATSON, A. RAVVE, J. M. SUGIHARA, AND W. J. BURKE.....	903
Factors Influencing the Antibiotic Activity of Lupulon. YIN-CH'ANG CHIN, NAI-CH'U CHANG, AND HAMILTON H. ANDERSON.....	909
Lupulon and Humulon—Antibiotic Constituents of Hops. J. C. LEWIS, GORDON ALDERTON, J. F. CARSON, D. M. REYNOLDS, AND W. D. MACLAY.....	916
The Occurrence of Antibacterial Substances Active Against <i>Mycobacterium Tuberculosis</i> in Seed Plants. R. Y. GOTTSHALL, E. H. LUCAS, ARDETH LICKFELDT, AND J. M. ROBERTS.....	920
A Crystalline Antifungal Agent, Mycosubtilin, Isolated from Subtilin Broth. ROBERT B. WALTON AND H. BOYD WOODRUFF.....	924
The Antifungal Activity of Antibiotic XG. GLADYS L. HOBBY, PETER P. REGNA, NANCY DOUGHERTY, AND WILLIAM E. STIEG.....	927
Neomycin—Production and Antibiotic Properties. SELMAN A. WAKSMAN, HUBERT A. LECHEVALIER, AND DALE A. HARRIS.....	934
Diffusion Plate Assay for Chloramphenicol and Aureomycin. W. A. RANDALL, A. KIRSHBAUM, J. K. NIELSEN, AND D. WINTERMERE.....	940
Chloramphenicol (Chloromycetin), an Antibiotic. Pharmacological and Pathological Studies in Animals. O. M. GRUHZIT, R. A. FISKEN, T. F. REUTNER, AND EDITH MARTINO.....	943
Susceptibility of Micro-Organisms to Chloramphenicol (Chloromycetin). I. W. MCLEAN, JR., J. L. SCHWAB, A. B. HILLEGAS, AND A. S. SCHLINGMAN.....	953
Preliminary Report on the Use of Chloramphenicol (Chloromycetin) in the Treatment of Acute Gonorrheal Urethritis. J. E. SMADEL, C. A. BAILEY, AND D. S. MANKIKAR.....	964

The Beneficial Effect of Chloromycetin in Brucellosis. THEODORE E. WOODWARD, JOSEPH E. SMADL, WILLIAM A. HOLBROOK, AND WILLIAM T. RABY.....	968
The Effect of Aureomycin Against <i>Bacterium Tularensis</i> . JOHN C. RANSMER.....	977
The Concentration of Aureomycin in Urine and Cerebrospinal, Pleural and Ascitic Fluids after Oral and Intravenous Administration. HARRY F. DOWLING, MARK H. LEPPER, ESTON R. CALDWELL, JR., RICHARD L. WHELTON, AND ROBERT L. BRICKHOUSE.....	983
Clinical Experience with Aureomycin. JOHN C. HARVEY, GEORGE S. MIRICK, AND ISABELLE G. SCHAUB.....	987
The Clinical Evaluation of Aureomycin. HENRY BRAINERD, EDWIN H. LENNETTE, GORDON MEIKLEJOHN, HENRY B. BRUYN, JR., AND WILLIAM H. CLARK.....	992
Further Experiences with Intravenous Aureomycin Therapy. A Study of 116 Cases. MURRAY SANDERS, JOHN M. RUMBALL, CYRIL SOLOMON, M. GONZALEZ SORET, AND NELDA I. RICCI.....	1006
Purity Studies on Polypeptide Antibiotics: Bacitracin. LYMAN C. CRAIG, J. DELAFIELD GREGORY, AND GUY T. BARRY.....	1014
A Comparative Study of the Renal Damage Produced in Mice by Various Lots of Bacitracin. LAWRENCE W. SMITH, FRED H. SCHULTZ, JR., WALLACE L. OTT, AND HARTZELL G. PAYNE..	1018
Chemical Studies on Polymyxin B. PETER P. REGNA, I. A. SOLOMONS, BERNARD K. FORSCHER, AND ALBERT E. TIMRECK.....	1022
Laboratory and Clinical Studies of Polymyxin B and E. EDWIN J. PULASKI, HINTON J. BAKER, MILTON L. ROSENBERG, AND JAMES F. CONNELL, JR.....	1028
The Pharmacology of Circulin. MILTON J. VANDER BROOK AND MARILYN T. RICHMOND....	1032
Preparation of a Modified Subtilin Suitable for the Treatment of Tuberculosis and Other Infections in Animals. A. J. SALLE AND GREGORY J. JANN.....	1036
Abstracts.....	1039

NUMBER 5, PART 2, SEPTEMBER, 1949

The Serum Proteins in Infectious Mononucleosis. Electrophoretic Studies. KENNETH STERLING.....	1057
Evidence that Renal Sodium Excretion by Normal Human Subjects is Regulated by Adrenal Cortical Activity. ALEXANDER LEAF AND WILLIAM T. COUTER.....	1067
Some Effects of Variation in Sodium Intake and of Different Sodium Salts in Normal Subjects. ALEXANDER LEAF, WILLIAM T. COUTER, AND L. H. NEWBURGH.....	1082
Further Studies with the Salt Tolerance Test in Normal Individuals and in Patients with Adrenal Cortical Hyperfunction. L. J. SOFFER, J. L. GABRILOVE, AND M. D. JACOBS...	1091
The Action of Streptococcal Desoxyribose Nuclease (Streptodornase), <i>In Vitro</i> and on Purulent Pleural Exudations of Patients. SOL SHERRY, ALAN JOHNSON, AND WILLIAM S. TILLET.....	1094
Studies with Radioactive Iodized Fat. I. Preparation of Radioactive Fat with Observations on the Absorption of Fat following Subcutaneous and Intraperitoneal Injection in Dogs. ALEXANDER M. RUTENBURG, ARNOLD M. SELIGMAN, AND JACOB FINE.....	1105
Studies with Radioactive Iodized Fat. II. The Tissue Distribution of Emulsified Fat following Intravenous Administration. ALEXANDER M. RUTENBURG, ARNOLD M. SELIGMAN, AND JACOB FINE.....	1110
The Measurement of Extracellular Fluid in Man by Means of a Constant Infusion Technique. IRVING L. SCHWARTZ, DAVID SCHACHTER, AND NORBERT FREINKEL.....	1117
Studies of the Role of the Liver in Human Carbohydrate Metabolism by the Venous Catheter Technic. II. Patients with Diabetic Ketosis, Before and After the Administration of Insulin. PHILIP K. BONDY, WALTER LYON BLOOM, VIRGINIA S. WHITNER, AND BETTY W. FARRAR.....	1126

Studies of the Pulmonary Circulation. I. The Circulation Time from the Pulmonary Artery to the Femoral Artery and the Quantity of Blood in the Lungs in Normal Individuals. RICHARD V. EBERT, CRAIG W. BORDEN, HERBERT S. WELLS, AND RUSSELL H. WILSON. . .	1134
Studies of the Pulmonary Circulation. II. The Circulation Time from the Pulmonary Artery to the Femoral Artery and the Quantity of Blood in the Lungs in Patients with Mitral Stenosis and in Patients with Left Ventricular Failure. CRAIG W. BORDEN, RICHARD V. EBERT, RUSSELL H. WILSON, AND HERBERT S. WELLS.	1138
Maturation of Renal Function in Childhood: Clearance Studies. MITCHELL I. RUBIN, ERIKA BRUCK, AND MILTON RAPOPORT.	1144
The Cerebral Blood Flow in Male Subjects as Measured by the Nitrous Oxide Technique. Normal Values for Blood Flow, Oxygen Utilization, Glucose Utilization, and Peripheral Resistance, with Observations on the Effect of Tilting and Anxiety. PERITZ SCHEINBERG AND EUGENE A. STEAD, JR.	1163
Serological Studies in Rheumatic Fever. II. Serum Complement in the Rheumatic State. EDWARD E. FISCHER, RUTH H. PAULI, AND JACK LESH.	1172
Evaluation of Therapeutic Substances Employed for the Relief of Bronchospasm. V. Adrenergic Agents. ELLIOTT BRESNICK, JOHN F. BEAKEY, LEON LEVINSON, AND MAURICE S. SEGAL.	1182
Evaluation of Therapeutic Substances Employed for the Relief of Bronchospasm. VI. Aminophylline. MAURICE S. SEGAL, LEON LEVINSON, ELLIOTT BRESNICK, AND JOHN F. BEAKEY.	1190
Chloramphenicol (Chloromycetin) in the Treatment of Tsutsugamushi Disease (Scrub Typhus). JOSEPH E. SMADEL, THEODORE E. WOODWARD, HERBERT L. LEY, JR., AND RAYMOND LEWTHWAITE.	1196
Changes in Liver Glycogen Studied by the Needle Aspiration Technic in Patients with Diabetic Ketosis. With a Method for the Estimation of Glycogen from Histologic Preparations. PHILIP K. BONDY, WALTER H. SHELDON, AND LILLIAN D. EVANS.	1216
Comparisons of the Distribution of Radioactive Iodine in Serum and Urine in Different Levels of Thyroid Function. ROBERT H. WILLIAMS, HERBERT JAFFE, AND BEATRICE BERNSTEIN	1222
Effects of Adrenocorticotrophic Hormone on Neuro-Muscular Function in Patients with Myasthenia Gravis. CLARA TORDA AND HAROLD G. WOLFF.	1228
The Serum Proteins in Portal Cirrhosis under Medical Management. Electrophoretic Studies. KENNETH STERLING, WILLIAM E. RICKETTS, JOSEPH B. KIRSNER, AND WALTER L. PALMER.	1236

NUMBER 6, PART 1, NOVEMBER, 1949

SYMPOSIUM ON RADIOACTIVE ISOTOPES

Foreword.	i
Production and Availability of Radioisotopes. PAUL C. AEBERSOLD.	1247
The Use of Stable Isotopes in Biological and Medical Research. IRVING M. LONDON.	1255
Dosage Determination in the Use of Radioactive Isotopes. LEONIDAS D. MARINELLI.	1271
Personnel Protection in the Use of Radioactive Isotopes. G. FAILLA.	1281
Biological Hazards and Toxicity of Radioactive Isotopes. AUSTIN M. BRUES.	1286
Equations for Tracer Experiments. A. K. SOLOMON.	1297
The Therapeutic Application of Radioactive Phosphorus with Special Reference to the Treatment of Primary Polycythemia and Chronic Myeloid Leukemia. HYMER L. FRIEDEL AND JOHN P. STORAASLI.	1308
Radioactive Phosphorus in Studies on the Metabolism of Normal and Neoplastic Tissues. A. MARSHAK.	1324

The Use of Radioactive Iodine in Studying the Pathologic Physiology of Thyroid Disease. RULON W. RAWSON.....	1330
The Use of Radioiodine in Physiological and Clinical Studies on the Thyroid Gland. M. S. RABEN AND E. B. ASTWOOD.....	1347
Radioiodine Therapy in Graves' Disease: A Review. MAYO H. SOLEY AND NADINE FOREMAN	1367
The Uptake and Elimination of Krypton and Other Inert Gases by the Human Body. C. A. TOBIAS, H. B. JONES, J. H. LAWRENCE, AND J. G. HAMILTON.....	1375

NUMBER 6, PART 2, NOVEMBER, 1949

The Hemodynamic Effects of Hypotensive Drugs in Man. II. Dihydroergocornine. EDWARD D. FREIS, JOSEPH R. STANTON, JULIUS LITTER, JAMES W. CULBERTSON, MEYER H. HALPERIN, F. CORBIN MOISTER, AND ROBERT W. WILKINS.....	1387
The Effect of a Cation Exchange Resin on Electrolyte Balance and its Use in Edematous States. LESLIE IRWIN, EUGENE Y. BERGER, BENJAMIN ROSENBERG, AND ROSLYN JACKENTHAL	1403
Carbon Tetrachloride Poisoning in Man. I. The Mechanisms of Renal Failure and Recovery. JONAS H. SIROTA.....	1412
Normal Respiratory and Circulatory Pathways of Adaptation in Exercise. ROBERT A. BRUCE, FRANK W. LOVEJOY, JR., RAYMOND PEARSON, PAUL N. G. YU, GEORGE B. BROTHERS, AND TULIO VELASQUEZ.....	1423
Variability of Respiratory and Circulatory Performance During Standardized Exercise. ROBERT A. BRUCE, RAYMOND PEARSON, FRANK W. LOVEJOY, JR., PAUL N. G. YU, AND GEORGE B. BROTHERS.....	1431
Studies in Methionine Metabolism. II. Fasting Plasma Methionine Levels in Normal and Hepatopathic Individuals in Response to Daily Methionine Ingestion. LAURANCE W. KINSELL, HAROLD A. HARPER, GRACE K. GIESE, SHELDON MARGEN, DAVID P. MCCALLIE, AND JEAN R. HESS.....	1439
Protamine (Salmine) Sulphate, Heparin, and Blood Coagulation. A. FRANK PORTMANN AND WILLIAM D. HOLDEN.....	1451
The Effect of Theophylline Ethylene Diamine on Renal Function in Control Subjects and in Patients with Congestive Heart Failure. JAMES O. DAVIS AND NATHAN W. SHOCK.....	1459
Electrophoretic Studies of the Serum Proteins in Biliary Cirrhosis. KENNETH STERLING AND WILLIAM E. RICKETTS.....	1469
Electrophoretic Studies of the Serum Proteins in Virus Hepatitis. WILLIAM E. RICKETTS AND KENNETH STERLING.....	1477
Influence of the Adrenal Cortex on Body Water Distribution and Renal Function. MARIO GAUDINO AND MARVIN F. LEVITT.....	1487
Electrolyte Balances in a Male Infant with Adrenocortical Insufficiency and Virilism. The Effect of Desoxycorticosterone Acetate and Salt Therapy with Special Reference to Potas- sium. HENRY L. BARNETT AND HELEN MCNAMARA.....	1498
Thrombin Formation. II. The Effects of Lysin (Fibrinolysin, Plasmin) on Prothrombin, Ac-Globulin and Tissue Thromboplastin. JESSICA H. LEWIS, ANN C. HOWE, AND J. H. FERGUSON.....	1507
Prothrombin Consumption, Serum Prothrombic Activity and Prothrombin Conversion Accel- erator in Hemophilia and Thrombocytopenia. BENJAMIN ALEXANDER AND GRETA LANDWEHR.....	1511
Multiple-Balloon-Kymograph Recording of Intestinal Motility in Man with Observations on the Correlation of the Tracing Patterns with Barium Movements. WILLIAM P. CHAPMAN AND WILLIAM L. PALAZZO.....	1517
Renal Tubular Secretion of Potassium in Man. ALEXANDER LEAF AND AUGUSTO A. CAMARA	1526

Circulatory Dynamics Before and After Exercise in Subjects With and Without Structural Heart Disease during Anxiety and Relaxation. IAN P. STEVENSON, CHARLES H. DUNCAN, AND HAROLD G. WOLFF.	1534
The Role of Oxygen in the Regulation of Erythropoiesis. Depression of the Rate of Delivery of New Red Cells to the Blood by High Concentrations of Inspired Oxygen. JOHN C. TINSLEY, JR., CARL V. MOORE, REUBENIA DUBACH, VIRGINIA MINNICH, AND MOISES GRINSTEIN.	1544
The Relationship Between Serum Lipids and Skin Xanthomata in Eighteen Patients with Primary Biliary Cirrhosis. EDWARD H. AHRENS, JR., AND HENRY G. KUNKEL.	1565
The Relationship Between Serum Lipids and the Electrophoretic Pattern, with Particular Reference to Patients with Primary Biliary Cirrhosis. HENRY G. KUNKEL AND EDWARD H. AHRENS, JR.	1575
Index to Volume XXVIII.	1581

trations of sodium and chloride, the chief electrolytes of extracellular fluid and serum, were not increased above the usual range of isotonicity (Table II, at zero point of Period I). As a matter of fact, the concentrations of sodium were abnormally low in five patients. In all of these and in one other the concentrations of chloride in serum, taking 97 m.eq. per liter as the lower limit of normal, were also decreased (24). In view of the dehydration described above, it is immediately apparent that these patients had lost, in keeping with the findings in other series, considerable amounts of the chief extracellular electrolytes, con-

comitant with the losses of electrolytes. It is known, of course, that analysis and abnormally low levels alone been lost, potassium frequently coexist (1), would have been, not, however, an invariable

During the first analysis can occur with normal 0.9 per cent saline of potassium (35) and our concentrations of sodium pronounced hypopotassemia patients (Table II, evident paralysis. It can be drawn as to definite hypernatremia and hyponatremia deficits of potassium. In subject R. J., for example, shown, for with normal concentrations, the sodium potassium increased to 161.6 m.eq. per liter and hydrocortis-

TABLE II
Body weight and analyses of blood and serum during treatment of diabetic acidosis and coma

Patient (age—sex)	Period	Time from start	Body* wtg.	Blood*		Serum*			
				NPN	Sugar	HCO ₃	Cl	Na	K
		hours	kgm.	mgm. %	mgm. %	m. eq./liter	m. eq./liter	m. eq./liter	m. eq./liter
T.W. (26F)	I pre-KCl	0		—†	572	5.5	101.9	142.3	4.0
	II KCl p.o.	0-25		40	112	11.8	127.8	157.6	2.8
	III post-KCl	25-48		37	—		124.9	153.1	5.6
		48-70	60.8	34	—	16.4	110.2	144.1	5.4
M.S. (15F)	I pre-KCl	0		65	667	9.3	77.6	118.6	3.4
	II KCl p.o.	0-17		36	104	17.4	107.7	144.6	3.9
	III post-KCl	17-39		26	139	24.0	109.8	143.2	5.4
		39-61	41.5	26	259	23.4	102.8	144.8	5.6
J.K. (18F)	I pre-KCl	0	50.0	32	600	8.1	94.1	135.5	5.4
	II KCl p.o.	0-20	53.9	21	145	19.0	97.8	140.5	5.0
	III post-KCl	20-67	53.4	27	235	23.4	98.8	144.4	5.4
		67-72	53.4	33	302	22.7	98.1	137.8	6.0
W.S. (14M)	I pre-KCl	0	36.2	39	470	6.7	96.3	129.9	5.5
	II KCl p.o.	0-12		21	70	14.7	114.9	149.6	4.1
	III post-KCl	12-44		27	362	22.4	98.9	144.8	5.5
		44-68	38.1	30	181	27.2	94.4	151.2	4.4
L.D. (15M)	I pre-KCl	0	38.7	33	1189	4.1	96.5	124.2	4.0
	II KCl p.o.	0-23	38.3	24	51	11.2	109.3	143.9	1.8
	III post-KCl	23-41	40.9	28	328	15.7	101.8	137.3	3.6
		41-65	38.4	35	352	24.0	98.0	142.5	3.0
R.J. (14M)	I pre-KCl	0	34.9	103	1395	10.4	101.9	142.1	3.7
	II KCl p.o.	0-19		79	230	20.6	137.3	161.6	2.0
	III post-KCl	19-44		49§	118	21.3	123.5	143.9	4.1
		44-68	47.2	41§	288	25.4	108.9	133.5	3.8
D.C.(a) (16F)	I pre-KCl	0	52.7	65	582	4.8	89.9	122.7	6.5
	II KCl p.o.	0-23	53.4	28	171	15.1	111.5	155.9	3.0
	III post-KCl	23-59	52.8	32	140	26.9	95.5	133.2	4.3
		59-83	54.0	37	167	29.2	98.1	139.1	4.1
D.C.(b) (16F)	I pre-KCl	0	50.0	67	786	6.8	91.4	125.3	6.7
	II KCl i.v.	0-19	53.9	28	163	15.4	117.1	145.6	3.3
	III post-KCl	19-51	50.9	29	149	26.1	92.1	140.8	5.7
		51-84	50.9	34	55	28.7	94.1	141.0	4.7

* Values as recorded refer to body weight, blood, or serum observed on admission (0 hour), and at the end of each individual period.

† Assumed to be 60 for purposes of calculating balances of cell nitrogen.

‡ Not obtained. Average value of the seven other cases used in calculating concentrations in extracellular water, 91

§ NPN drawn before end of period; lower value therefore assumed in calculations.

Circulatory Dynamics Before and After Anesthesia in Healthy adults. Heart Disease during Anesthesia. What this hyper- AND HAROLD G. WOLFF. ious. The find- The Role of Oxygen in the Regulation of the Metabolism in most instances of New Red Cells to the Blood. on could have been TINSLEY, JR., CARL V. Would have received GRINSTEIN. olution. During the The Relationship Between the Serum Chloride and the Metabolism, or even below, physiological The Relations. The serum sodium levels during Refers I and III in general showed a similar H. A. in no instance, in contrast to the chlor- Index to did hyponatremia appear.

arbonate: The markedly lowered concentration of serum bicarbonate present on admission toward or to normal during therapy in

all patients. The development of hyperchloremia, which was as has already been noted an almost invariable occurrence, did not prevent these increases in bicarbonate. However, it is not possible to answer unequivocally from these data the question as to whether or not hyperchloremia repressed the rise of serum bicarbonate. It is clear, for example, that the increase during the first period in serum bicarbonate level of 10.9 m.eq. per liter in 20 hours in patient J. K. who did not develop hyperchloremia is quite comparable to the 10.2 m.eq. per liter increase in 19 hours in the patient with the most pronounced hyperchloremia (R. J.). On the other hand, the possibility that hyperchloremia prolonged the acidosis in patient T. W. can by no means be excluded. At 70 hours the bicarbonate concentration was still only

TABLE III

*Balances of electrolytes, nitrogen, and carbohydrate during treatment of diabetic acidosis and coma**

Pt.	Period	External balance†					Extracellular balance			Intracellular balance‡		
		Cl	Na	K	N	CHO	Fluid	Na	K	N	Na	K
		<i>m. eq.</i>	<i>m. eq.</i>	<i>m. eq.</i>	<i>gms.</i>	<i>gms.</i>	<i>liters</i>	<i>m. eq.</i>	<i>m. eq.</i>	<i>gms.</i>	<i>m. eq.</i>	<i>m. eq.</i>
T.W.	I	+1533	+1616	+ 11	- 7.6	+289	+9.5	+1561	+15	- 0.5	+ 56	- 3
	II	+ 434	+ 124	+450	+ 4.7	+257	+3.5	+ 463	+69	+ 5.8	-339	+367
	III	- 375	- 209	- 5	+ 1.2	+240	-0.3	- 228	- 6	+ 2.3	+ 19	- 5
J.	I	+ 638	+ 674	- 48	-10.4	+108	+3.7	+ 672	+17	- 2.6	+ 2	- 59
	II	- 102	- 101	+108	- 2.5	+ 74	-1.0	+ 165	+10	+ 0.2	- 64	+ 98
	III	- 90	- 68	+ 22	+ 1.0	+ 43	-0.2	- 6	+ 1	+ 1.0	- 62	+ 19
K.	I	+ 503	+ 491	- 75	- 8.6	- 16	+4.4	- 650	+19	- 5.0	+159	- 82
	II	- 38	- 122	+297	+ 3.1	+278	-0.5	- 20	+ 2	+ 1.2	-102	+292
	III	- 16	+ 2	+ 8	+ 2.1	+ 84	0	+ 86	+ 8	+ 0.2	- 88	0
W.S.	I	+ 443	+ 489	- 53	- 6.2	+ 49	+2.7	- 494	+ 3	- 1.9	+ 5	- 52
	II	- 418	- 136	+139	+ 7.2	+ 96	-2.5	+ 398	- 3	+ 5.9	-262	+128
	III	+ 15	- 170	+ 1	+ 0.1	+ 52	+0.4	- 100	- 4	- 0.6	+270	+ 7
L.D.	I	+ 195	+ 284	- 67	- 7.1	+ 76	+1.0	+ 249	-12	- 4.8	+ 35	- 44
	II	+ 148	+ 61	+344	+ 9.8	+133	+1.9	+ 205	+19	+ 8.8	+144	+304
	III	- 280	- 130	+107	+10.2	+188	-2.3	- 274	-12	+ 8.4	+144	+ 99
R.J.	I	+ 841	+ 896	- 25	- 1.7	+ 76	+4.2	+ 772	- 1	+ 3.7	+124	- 34
	II	+ 146	- 74	+262	- 6.1	+ 28	+2.3	- 138	+28	+ 2.6	-212	+228
	III	- 236	- 136	- 25	-10.0	- 21	-0.4	- 174	+ 4	- 8.6	+ 38	- 9
D.C.(a)	I	+ 655	+ 730	-106	-14.2	+ 57	+4.1	- 865	-17	- 1.5	+135	- 86
	II	- 517	- 529	+239	- 9.7	- 62	-3.0	+ 657	+ 3	-11.0	-128	+262
	III	- 65	+ 65	+ 38	+ 1.0	+ 69	-0.8	+ 62	- 5	- 0.8	-127	+ 45
D.C.(b)	I	+ 892	+ 798	-125	- 5.4	+146	+5.3	- 909	- 9	+ 7.3	-111	-116
	II	- 355	- 392	+285	+ 2.7	+135	+0.2	- 57	+31	+ 2.4	-335	+248
	III	+ 1	+ 33	+ 31	+ 4.7	+125	-0.6	+ 33	-14	+ 3.1	+ 66	+ 38

* Balance data are expressed per individual period rather than cumulatively.

† Corrected for the electrolytes and nitrogen withdrawn in serum samples. Stools, when passed, were formed and not analyzed. Small amounts of vomitus in T.W. and 240 cc. in L.D. in the pre-KCl periods were not analyzed.

‡ The K balances represent changes in excess of the transfers associated with the anabolism and catabolism of proteins. Similar treatment of the Na data does not significantly affect the results. Changes in the NPN have been taken into account in calculating the balance of cell nitrogen (17-19).

16.4 m.eq. per liter, even though hyperglycemia and acetonuria had disappeared.

3. *Potassium*: Abnormally high levels of potassium (6.5 and 6.7 m.eq. per liter) were noted on admission twice in the same patient, D. C. (a) and D. C. (b) in Table II. In the other six cases the admission values ranged from 3.4 to 5.5. During the initial period of therapy in which no potassium was given a dramatic fall of serum potassium to abnormally low levels was observed in patients T. W., L. D., R. J., D. C. (a) and D. C. (b). Though the intermediate points are not recorded the values given are actually the lowest concentrations observed in each patient before the start of KCl *per os* or parenterally. Only one of the subjects, R. J., developed obvious muscular paralysis, with inability to move his extremities. As a group, however, the patients were still too ill at the time of most marked hypopotassemia to permit clear differentiation between generalized asthenia and partial paralysis.

The administration of KCl without exception increased the concentration of serum potassium. The highest recorded level, 5.7 m.eq. per liter, at the end of KCl therapy was observed in the one patient, D. C. (b), who received the salt intravenously. However, a comparable or even greater increment from 2.8 to 5.6 m.eq. per liter occurred in patient T. W. who took the KCl *per os*. It is to be noted that the muscular paralysis of subject R. J. cleared promptly following the oral administration of the potassium salt. None of the subjects in this series developed gastrointestinal distress or diarrhea, even though the KCl was administered in capsules or intravenously as rapidly as 1 gm. per hour.

C. Balances of carbohydrate, nitrogen, and electrolytes

Results of calculations based on the data in Tables I and II have been recorded in Table III. During Period I the external balances indicated that, with but one exception, the patients had utilized or stored about one-half and in one instance as much as 96 per cent of the administered carbohydrate. Since the concentration of blood sugar fell in each patient during this interval, it is obvious that the balance was even more positive. Similarly, in the subsequent two periods of

observation, valuated. It is known, of course, predominate analysis and abnormally low levels

Without potassium frequently coexist (1, excreted nitrogens not, however, an invariable gen intake (Ta-analysis can occur with normal of this negative t of potassium (35) and our a decrease in the et pronounced hypopotassemia present on adt. evident paralysis.

has been included in series can be drawn as to gen balance, six of the eight patients deficits of cell nitrogen in Period I (Table I) shown, for the KCl and post-KCl periods, when potassium were taking milk, the trend was reversed, corticosteroid balances of cell nitrogen, with but potassium in- exceptions, became positive.

In so far as the balances of electrolytes it is of concerned during the pre-KCl period, 26 years retained large amounts of chloride on admission a While KCl was being administered, characterized subsequent interval the external balance changes sodium and of chloride were more often still than positive (Table III). However, not after the negative balances was of sufficient magnitude to cancel entirely the earlier retention of sodium or chloride. The largest positive balances of sodium and of chloride, more than 1500 m.eq. as to each ion for the three periods, were recorded in T.W., even though this patient was associated with normal concentrations of sodium and chloride. This finding is to be contrasted with the relatively small positive balances of these ions in patient L. D. despite initial hypochloremia and hyponatremia. It should be emphasized, however, that irrespective of the initial concentrations of sodium and chloride in the serum all patients retained these two electrolytes in greater or less proportion. Again, as in the interpretation of the positive balances of extracellular water, this can be taken as evidence of replacement of deficits but it is possible that some of it represents over-treatment. The retained sodium was disparately distributed between the extracellular and cellular fluid. Most of it, and frequently all, remained in the extracellular phase (Table III).

In contrast to the positive balances of sodium, all but one patient lost considerable quantities of body potassium during the first period of treatment. This one patient, T. W., had received, however, a moderate amount of potassium during this interval. During the second period of th-

Circulatory Dynamics Injected the Heart Disease during III under AND HAROLD G. Went of the ad- The Role of Oxygen in the positive ex- of New Red Cells the first case TINSLEY, JR., Ceq. per kgm. of GRINSTEIN. . . . retention in pro- The Relationship Ceq. per kgm., was Primary Biliary It is to be noted that The degrees of potassium retention were patient D. C. during two separate ad- If coma irrespective of the route of ad- m. In the first instance, D. C. (a), the administered orally, and in the second, intravenously. Furthermore, the per retention of potassium given parenterally Subject was of the same order of magnitude served on the average in the six other treated *per os*. As might be predicted, r portion of the retained potassium en- e cellular phase (Table III). The cell es of potassium during the second period, rected for the intracellular balances of nitro- n, ranged from + 98 to + 406 m.eq. In some the experiments sodium left the cells as potas- tered, but this was by no means an in- e finding. The failure to observe recipro- changes of these two ions more often may been related in part to the lag in the extru- of sodium reported by Conway and Hingerty owing replacement of experimentally induced potassium deficits (27). Given with these large positive balances of po- tassium it is not certain that the deficits had been completely corrected in all patients. As a matter of fact, in the third period, during which the intake was limited to milk and water, only one of the eight patients developed negative balances of cell potassium in excess of nitrogen. The others were still retaining a part or all of the potassium available to them in the milk ration.

DISCUSSION

The electrolyte, nitrogen, and carbohydrate studies in these patients again emphasize that considerable amounts of body constituents are lost during diabetic coma. The losses of body water, of extracellular electrolytes, of cell nitrogen, and of carbohydrate have been recognized and de- scribed (25, 26). The striking retention of potas-

sium administered to this series of patients points to the existence of concomitant deficits of this cation in the body cells. Such an interpretation is supported by the experiments of Tarail and Elkinton which showed that amounts of potassium as great as those employed in some of our studies were not retained in the body when given by mouth to healthy adults (28). Presumably such subjects do not have potassium deficits and hence the administered potassium is promptly excreted. Diabetic patients in acidosis or coma are, however, depleted of potassium. Several mechanisms appear to be involved in the development of this negative balance. It is obvious, for example, that patients in diabetic acidosis or coma are in negative nitrogen balance. This breakdown of tissue releases potassium bound to protein of cells (17). Similarly, potassium laid down with glycogen in the liver (29) is released from the cells during deglycogenation. These two processes, however, can account for only a small portion of the potassium deficit. Most of the deficit must represent, therefore, a decrease in the cell potassium present in excess of the nitrogen there. Several factors probably contribute to this loss of cell base. First, it is known that extracellular dehydration, *per se*, is associated with movements of potassium and of water out of the cells (17). Secondly, it has been shown that interruption of carbohydrate metabolism in the blood cell *in vitro*, either by refrigeration, exhaustion of glucose stores, or by addition of an inhibitor such as fluoride, is associated with a pouring out of cell potassium (30). Finally, the evidence to date suggests that as long as urine is being elaborated it contains potassium. This appears to be true even though deficits of cell potassium, and even extracellular hypopotassemia, are present (21, 28). Hence, with the diuresis characteristic of almost all patients in coma considerable amounts of potassium are lost. At present it is not clear to which of these mechanisms the major portion of the potassium deficit is to be ascribed.

The hypopotassemia of extracellular fluid which develops during treatment on a potassium-free regimen noted by other workers and by us stems from a number of sources. It is obvious from our data that it results in part from an expansion of body water with the administration of fluids,

and in part from a continued loss of potassium in urine. The negative balances of cell potassium recorded during the first period of therapy in seven of the eight patients exclude the possibility that reentry of this ion into cells played any significant role in the decrease in serum potassium. This does not mean, of course, that no potassium was deposited in the liver with reglycogenation, nor that none entered other cells. It merely indicates that these must have been subsidiary processes. Furthermore, the negative balances of potassium cannot be explained away as manifestations of transfers of chloride into cells. Such a movement of the reference ion could not cancel the negative cell balances, since even large alterations of the chloride space could account for only a few milliequivalents of potassium.

During the administration of potassium salts the major portion of the cation entered the cells. Only a small amount of it was laid down with nitrogen. There was no significant urinary loss of previously retained potassium in the post-KCl periods. As a matter of fact, in five of the experiments more potassium entered the cells. On the whole a smaller proportion of the administered potassium was retained during Period III suggesting that in the majority of the patients cell concentrations of this ion were completely or almost completely restored. It should be emphasized of course that the possibility that the potassium administered orally was incompletely absorbed cannot be excluded. Naturally this would alter the magnitude of the exchanges. However, the findings in control studies (28), and the absence of frequent or diarrheal stools in these patients, suggest that absorption was essentially complete. This is further supported by the finding that quite comparable data were obtained when KCl was given intravenously.

It is to be noted that positive cell balances of both potassium and sodium were recorded in patients who were admitted with essentially normal serum levels of these ions. These instances should again serve to emphasize the fact that the presence of normal concentrations of any particular electrolyte cannot be interpreted as evidence that the total amount is intact (31). This is of obvious importance in planning replacement therapy.

The physiological significance of these extensive potassium deficits can be as yet partially and

only indirectly evaluated. It is known, of course, that muscular paralysis and abnormally low levels of *extracellular* potassium frequently coexist (1, 2, 32-34). This is not, however, an invariable association, since paralysis can occur with normal serum concentrations of potassium (35) and our own data indicate that pronounced hypopotassemia can occur without evident paralysis.

At present only analogies can be drawn as to possible deleterious effects of these deficits of *intracellular* potassium. It has been shown, for example, that the depletion of cellular potassium produced by over-dosage with desoxycortosterone acetate or by restriction of potassium intake is associated with degenerative changes in the myocardium (36, 37). In this respect it is of interest that in our series patient T. W., 26 years of age and previously well, had on admission a grossly abnormal electrocardiogram, characterized by markedly inverted T waves. These changes compatible with myocardial damage were still present one month later, and cleared only after an additional month of convalescence. It may have been more than chance, therefore, that this occurred in the patient who apparently had developed the greatest deficits prior to therapy.

No adequate statistics are yet available as to whether replacement of potassium deficits may mitigate the considerable mortality still associated with diabetic acidosis and coma (26, 38). The favorable results of Govan and Darrow in decreasing the fatality rate of infantile diarrhea by means of parenteral and oral potassium therapy suggest that a similar beneficial response may be expected in diabetic coma (39). In testing this form of therapy, however, unremitting care must be taken to avoid deaths from potassium poisoning (40, 41). At present it would seem reasonable, therefore, to limit the administration of potassium salts to the oral route, or to give only dilute solutions intravenously. An adequate urine volume and constant electrocardiographic surveillance are essential.

SUMMARY AND CONCLUSIONS

Patients recovering from diabetic acidosis or coma retained considerable amounts of potassium administered as KCl, in addition to water, sodium, chloride, carbohydrate, and nitrogen. Comparable positive balances of this cation were re-

corded in one subject during two separate admissions, in one of which KCl was given by mouth and in the other intravenously. In every study potassium entered cells far in excess of amounts which could be ascribed to changes in cell protein. The possible physiological significance of these findings has been discussed.

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SIMULTANEOUS MEASUREMENT OF EFFECTIVE RENAL BLOOD FLOW AND CARDIAC OUTPUT IN RESTING NORMAL SUBJECTS AND PATIENTS WITH ESSENTIAL HYPERTENSION¹

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Unpublished data of Bradley and associates (1) indicate that about 25 to 30 per cent of the cardiac output, as measured by the ballistocardiograph (2), perfuses the kidney of healthy adults at rest. This figure has been reported in a review on renal physiology (3). Levy and Blalock (4) obtained an average value of 18.5 per cent in unanesthetized dogs, using the catheterization technique or heart puncture and the direct Fick principle for the determination of the cardiac output. When normal human values for the resting cardiac output obtained by the direct Fick procedure (5, 6) are divided into the normal human values for effective renal blood flow (7), the resulting effective renal fraction is about 19 to 20 per cent.

The present investigation was undertaken to make these measurements simultaneously in a series of normal subjects and in patients with essential hypertension, using the cardiac catheterization technique (8, 9) and the direct Fick principle for the measurements of the cardiac output.

METHODS

All studies were made during the morning at least 12 hours after the previous meal.

Effective renal blood flow was determined from the renal plasma clearance of p-aminohippurate or diodrast and from the hematocrit (10). At the same time, the glomerular filtration rate was measured by the renal plasma clearance of mannitol, inulin (10), or sodium

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thiosulfate (11). In about two-thirds of the cases the effective tubular excretory mass was determined from the excretion at high plasma concentrations of p-aminohippurate (T_{mpar}) or of diodrast (T_{md}) (10, 12). Urethral catheterization with saline and air washout was employed routinely.

Mixed venous blood for oxygen analysis was obtained through a cardiac catheter introduced into the pulmonary artery, the right ventricle, or failing these, the right auricle. The exact position in the auricle was checked fluoroscopically immediately before or after sampling to assure optimal placement (5, 10). After insertion of the cardiac and bladder catheters and placement of the indwelling femoral arterial needle,⁶ a rest period of 30 minutes ensued, following which the patient was familiarized with the noseclip, mouthpiece, and with bedside procedure. Several trial collections of expired air were obtained prior to the definitive collection, in order to minimize anxiety and to prove the stability of the ventilation, respiratory and heart rates. Arterial and mixed venous blood were drawn simultaneously under oil during the two-minute collection of expired air. Blood oxygen contents were determined in duplicate by the method of Van Slyke and Neill (13). Oxygen consumption was calculated from gas analysis of the expired air by the Haldane method (13).

Shortly before and after the above procedure, the arterial pressure was recorded optically by the Hamilton manometer (14), or the mean pressure was read directly from a calibrated aneroid manometer connected to the arterial needle through a short rubber tubing containing air which damped the pressure pulse almost completely. In the former case, the mean pressure was calculated from the weight of a representative sample cut out carefully from the photographic record. In a few subjects the arterial pressure was determined by cuff sphygmomanometry, the mean pressure being estimated as $P_m = P_D + 0.4 (P_s - P_D)$. The total peripheral resistance was calculated by dividing the mean arterial pressure by the cardiac output per 1.73 sq.m. and is expressed as dynes $cm.^{-2}$ sec. (absolute unit).

The metabolic rate was calculated from the minute volume of oxygen consumed and the assumed R.Q. of

⁶ Specially designed by Becton, Dickinson and Company.

TABLE I
Summary of data from control subjects

Patient	Age	Sex	Weight	Surface area	Hematocrit	Filtration rate			Effective renal blood flow	Temp	Filtration fraction	Respiratory rate	Ventilation rate	Oxygen intake	Metabolic rate	Arterial oxygen content	Oxygen difference	Cardiac index	Cardiac output	Heart rate	Mean arterial pressure	Peripheral resistance	Effective renal fraction
						Mannitol	Inulin	Sodium thiosulfate															
	yr.		kg.	sq.m.	per cent	cc./min.	cc./min.	cc./min.	cc./min.	mg./min.	mg./min.		L./min.	cc./min.	per cent	vol. per cent	vol. per cent	L./min.	L./min.	per min.	mm. Hg	A.U.	
P. M.	46	M	50.0	1.42	33		103.1		563	66.2	0.183	20	5.42	134	+1	15.3	3.1	4.29	7.42	80	83	950	0.132
J. D.	62	M	70.5	1.61	35	123.0			587		0.210	18	4.90	125	+6	14.7	3.2	3.89	6.73	80	88	1180	0.152
T. D.	48	M	54.0	1.61	34	67.2			594		0.113	9	6.83	144	+8	11.3	4.2	3.44	5.93	102	97	1310	0.150
E. O'D.	45	M	56.7	1.62	34	96.7			594		0.166	24	3.80	147	+10	12.6	4.2	3.49	6.01	100	101	900	0.111
M. S.	43	M	56.2	1.62	34			186.0	903	58.6	0.206	13	5.28	164	+5	15.7	2.7	4.68	8.10	76	74	680	0.162
F. F.	31	M	61.0	1.68	43	146.2			520		0.280	10	5.33	163	+23	12.6	4.0	4.67	8.10	70	95	1300	0.157
J. W.	35	M	64.0	1.72	45	130.0			754	75.0	0.173	16	4.31	133	-2	16.6	3.6	3.27	6.40	70	80	1000	0.204
J. S.	45	M	71.8	1.74	36	130.0			795		0.164	14	7.40	201	+3	16.3	3.5	4.09	7.03	63	91	960	0.170
N. W.	29	M	64.7	1.75	43			142.0	905	108.0	0.157	18	4.82	180	+35	14.1	4.6	3.92	6.78	70	78	1050	0.269
E. B.	44	M	71.5	1.87	33	114.3			434		0.264	15	4.33	134	+1	17.2	4.0	3.45	5.96	61	76	1020	0.125
R. W.	42	M	77.3	1.92	37	108.0			686		0.158	14	7.77	136	+2	14.3	2.9	4.69	8.11	78	85	835	0.139
T. K.	29	M	93.8	2.08	42		147.5		767	93.1	0.193	19	9.19	118	-11	13.2	3.1	3.81	6.60	78	86	1040	0.114
J. St.	52	F	40.0	1.28	29			94.5	489	48.5	0.193	20	6.05	137	+13	13.0	3.5	3.91	6.76	80	80	960	0.102
A. K.	49	F	55.0	1.52	42	94.5			489	63.9†	0.193	14	3.90	107	-14	18.7	4.4	2.44	4.22	88	100	1890	0.200
M. J.	37	F	64.1	1.60	34			104.5	555		0.188	25	4.62	121	-4	15.7	4.8	2.79	4.83	90	100	1655	0.144
E. K.	38	F	72.9	1.75	41			123.5	656	97.5	0.188	19	5.75	138	+10	14.7	3.7	3.75	6.49	88	103	1295	0.131
L. P.	21	F		1.48	35	128.5			806		0.159	17	5.67	154	+22	13.8	3.5	5.18	7.61	78	95	1000	0.140
S. W. C.	47	M	71.0	1.74	45		146.8		614	67.0	0.233	16	3.44	148	+12	19.2	5.5	5.17	8.95	104	116	1550	0.215
Aver.	41				36		121.5		651	75.4	0.190	17	5.43	144		15.1	3.7	4.07	7.05	81	92	1160	0.156

* Mean arterial pressure estimated from sphygmomanometer readings according to following formula: $P_m = P_D + 0.4(P_S - P_D)$.

† Temp determined two months after above studies at which time the filtration rate and renal plasma flow were essentially the same.

‡ In the calculation of the effective renal fraction the first cardiac output from each study was taken.

In columns 7 through 13 and 22 and 25 values are corrected to a surface area of 1.73 sq. m. In columns 16, 17 and 21 values are corrected to a surface area of 1.0 sq. m. Averages for columns 15 through 26 are calculated from values of first cardiac output determination.

TABLE II
Summary of data from hypertensive subjects

Patient	Age	Sex	Weight	Surface area	Hematocrit	Filtration rate			Effective renal plasma flow	Effective renal blood flow	T _{MPAH}	T _{MP}	Filtration fraction	Respiratory rate	Ventilation rate	Oxygen intake	Metabolic rate	Arterial oxygen content	Oxygen arterio-venous difference	Cardiac index	Cardiac output	Heart rate	Mean arterial pressure	Peripheral resistance	Effective renal fraction†
						Mannitol	Inulin	Sodium thiosulfate																	
J. S.	71	M	58.8	1.70	38		73.9		314	501			0.235	13	3.72	100	-20	17.2	5.0	2.00	3.46	58	150	3470	0.133
R. S.†	37	M	57.5	1.78	44				475	850	40.2		0.192	15	6.84	130	-4	19.5	5.2	2.35	4.11	61			
G. M.	50	M	73.0	1.81	40			83.5	457	769			0.186	18	5.50	163	+26	19.7	6.0	2.71	5.36	71	124	1850	0.158
H. H.†	49	M	75.0	1.83	48				758	1450			0.126	26	8.60	169	+27	18.8	4.2	4.06	7.02	76	135	2475	0.164
I. L.	47	M	86.5	1.94	32				706	1018	99.0		0.149	17	4.95	177	+33	13.0	4.2	5.85	10.10	86	120	950	0.207
S. B.†	36	M	85.9	1.98	49				382	744		28.7	0.226	18	5.32	181	+36	12.5	3.3	5.51	9.53	86			0.104
C. M.	39	F	46.0	1.34	21				405	524	41.0		0.161	29	9.34	146	+7	19.6	3.7	4.33	7.49	103	158	1690	0.111
L. M.	52	F	49.6	1.43	38			105.9	529	844	77.2		0.200	18	6.35	146	+16	9.7	4.3	3.39	5.86	106	166	2265	0.077
A. W.†	44	F	51.8	1.53	36				317	455		33.5	0.208	17	5.42	146	+23	15.0	1.8	8.19	14.15	96	123	695	0.061
C. B.†	53	F	50.3	1.54	31				465	675		36.4	0.194	16	5.11	140	+13	13.6	4.5	3.13	5.41	85	198	2930	0.080
M. R.	61	F	63.7	1.61	38				346	561	44.4		0.215	30	5.50	138	+10	10.0	3.7	3.72	6.43	127	1580	0.107	
M. B.†	33	F	65.5	1.68	41			74.3	436	740		42.1	0.224	22	5.99	132	+15	17.0	3.9	3.39	5.86	70	142	1940	0.109
J. McA.	51	F	69.5	1.72	46				391	713	70.6		0.238	15	5.59	140	+11	16.6	3.6	3.89	6.72	71	147	1750	0.118
M. D.	45	F	85.1	1.95	37				185	294			0.288	16	4.24	121	0	18.3	4.4	2.77	4.79	54	142	2370	0.149
M. Bl.	48	F	58.0	1.53	33				58	87	11.3		0.359	22	4.56	116	-6	16.0	5.2	2.26	3.92	72	132	2690	0.075
T. V.	59	M	64.5	1.78	46				267	495	42.9		0.311	12	5.83	161	+31	12.6	4.6	3.55	6.14	74	186*	2420	0.014
J. M.	60	M	61.4	1.57	38				573	924	79.7		0.106	20	4.05	135	+4	16.6	4.6	2.94	5.08	88	166*	2620	0.096
P.	43	F	82.0	1.85	47				400	754	74.9		0.219	24	4.27	186	+47	11.3	2.1	8.79	15.10	104	114*	604	0.061
M. deL.	48	F	55.9	1.51	34				222	338	37.6		0.358	22	4.93	183	+47	18.3	5.8	3.15	5.45	60	132*	1940	0.149
Aver.	48.7				39		79.8		405	670	57.9	36.2	0.221	19	4.15	172	+38	17.9	6.4	2.71	4.69	60	175*	1720	0.072
															4.57	162	+30	14.0	3.4	4.71	8.15	84			0.106

* Mean arterial pressure estimated from sphygmomanometer readings according to following formula: $P_m = P_D + 0.4(P_S - P_D)$.

† In the calculation of the effective renal fraction the first cardiac output from each study was taken.

‡ Results furnished by the courtesy of Dr. André Cournaud and associates.

In columns 7 through 13 and 22 and 25 values are corrected to a surface area of 1.73 sq. m. In columns 16, 17 and 21 values are corrected to a surface area of 1.0 sq. m. Averages for columns 15 through 26 are calculated from values of first cardiac output determination.

0.82. The observed R.Q. was not used for this purpose because the short collection period makes the CO_2 value too dependent on variations in the respiratory pattern, and therefore not a reliable measure of metabolic CO_2 production. The values of ventilation rate and oxygen consumption are corrected to saturation at 37°C . and prevailing barometric pressure.

CLINICAL SUBJECTS

All subjects were patients of the Third (New York University) Medical Division of Bellevue Hospital. The normal subjects are representative of the convalescent ward population of a large city hospital. Most of them had had an upper respiratory infection or acute alcoholism. There was one each with a diagnosis of mild anxiety neurosis, headache of unknown cause and without neurological signs, central nervous system degenerative disease, mild chronic cholecystitis, old quiescent small lung abscess, and central nervous system syphilis. In addition, six patients, convalescent from traumatic or hemorrhagic shock, who were studied 17 to 76 days after injury, are included in the present series by the kind permission of Drs. André Cournand, D. W. Richards, Jr., and their co-workers (15, 16). None of the normal subjects had any evidence of cardiovascular or renal disease. All had been afebrile and all but two were ambulatory for at least a week prior to study.

Most of the patients with essential hypertension were admitted to Bellevue from the New York University Hypertension and Nephritis Clinic for the express purpose of study. None presented evidence of cardiac insufficiency.

RESULTS

Tables I and II present the data from each subject. The more pertinent data are presented in the form of frequency diagrams in Figures 1 and 2.

The control and hypertensive groups are fairly comparable with respect to distribution of hematocrit, metabolic rate and pulse rate. This is considered essential for the comparison of the other values, in so far as deviations from accepted normal standards in these three might be expected to affect cardiac output and renal function. The hypertensive group averages 7.5 years older, and contains a higher proportion of women than the control group. The two groups are reason-

ably comparable as regards nutritional status and general health exclusive of hypertensive disease.

The cardiac output, expressed as liters per minute per square meter of body surface area, averages 4.07 in the control group as compared to 3.97 in the hypertensive group. The difference is not significant. This is in accord with the observations of previous investigators (17, 18).

Mean arterial pressure is, of course, markedly higher in the patients with essential hypertension. Since the cardiac output is about the same in both groups, the average calculated total peripheral resistance is elevated in the hypertensive group to about the same extent as is the arterial pressure.

Effective renal blood flow is within the normal range of plus or minus twice the standard deviation from the respective male and female means (7, 12) in 17 and below in only one of the control subjects. Ten of the hypertensive patients have renal blood flow within the normal range; eight are below it, and none exceeds it. Moreover, in all but one of the hypertensive patients the values are less than the normal mean.

The effective renal plasma flow is within the normal range in all of the control subjects, whereas it is below this range in eight of the 19 hypertensives.

The filtration rate is below the normal range in one control subject and above in another. In the rest, it is well within the normal range. Among the hypertensives, 11 fall below the range and none exceeds the normal mean.

Seven of the nine determinations of Tm_{PAH} made in the control group fall in the normal range reported by Chasis, Redish, Goldring, Ranges and Smith (12), while five of the ten determinations in hypertensive patients lie below this range. Diodrast Tm , measured in five hypertensives, is in the normal range in four and below normal in one.

The filtration fraction (filtration rate/effective plasma flow) averages 0.190 in the control group and 0.221 in the hypertensive group. The difference between normal and hypertensive patients is less striking than that observed by Goldring and Chasis (10).

The effective renal fraction (effective renal blood flow/cardiac output) was calculated from the average effective renal blood flow and from

NUMBER OF SUBJECTS

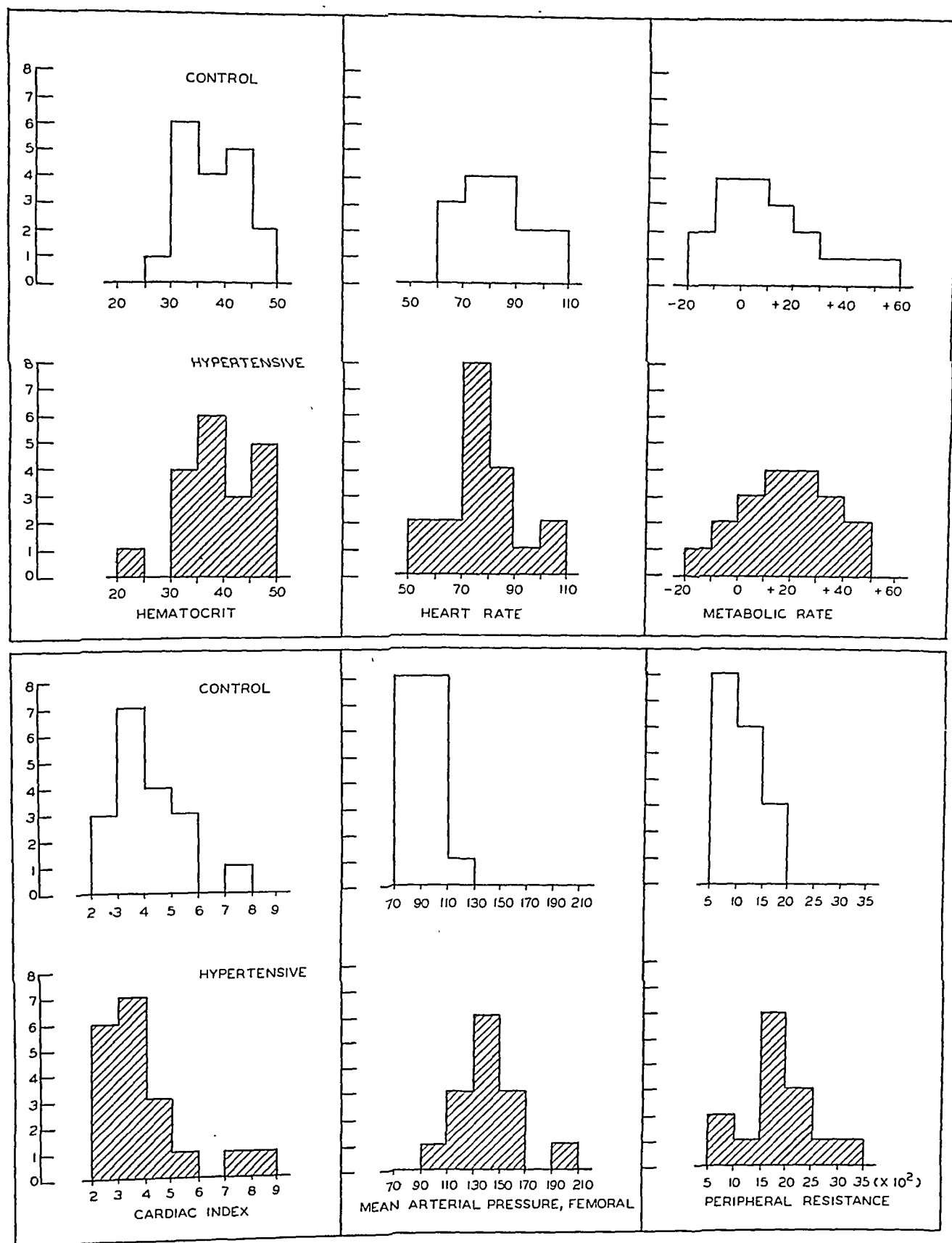


FIG. 1. FREQUENCY DISTRIBUTION OF OBSERVED PHYSIOLOGICAL VARIABLES IN CONTROL AND HYPERTENSIVE SUBJECTS

The values shown are those obtained in connection with the first cardiac output determination.

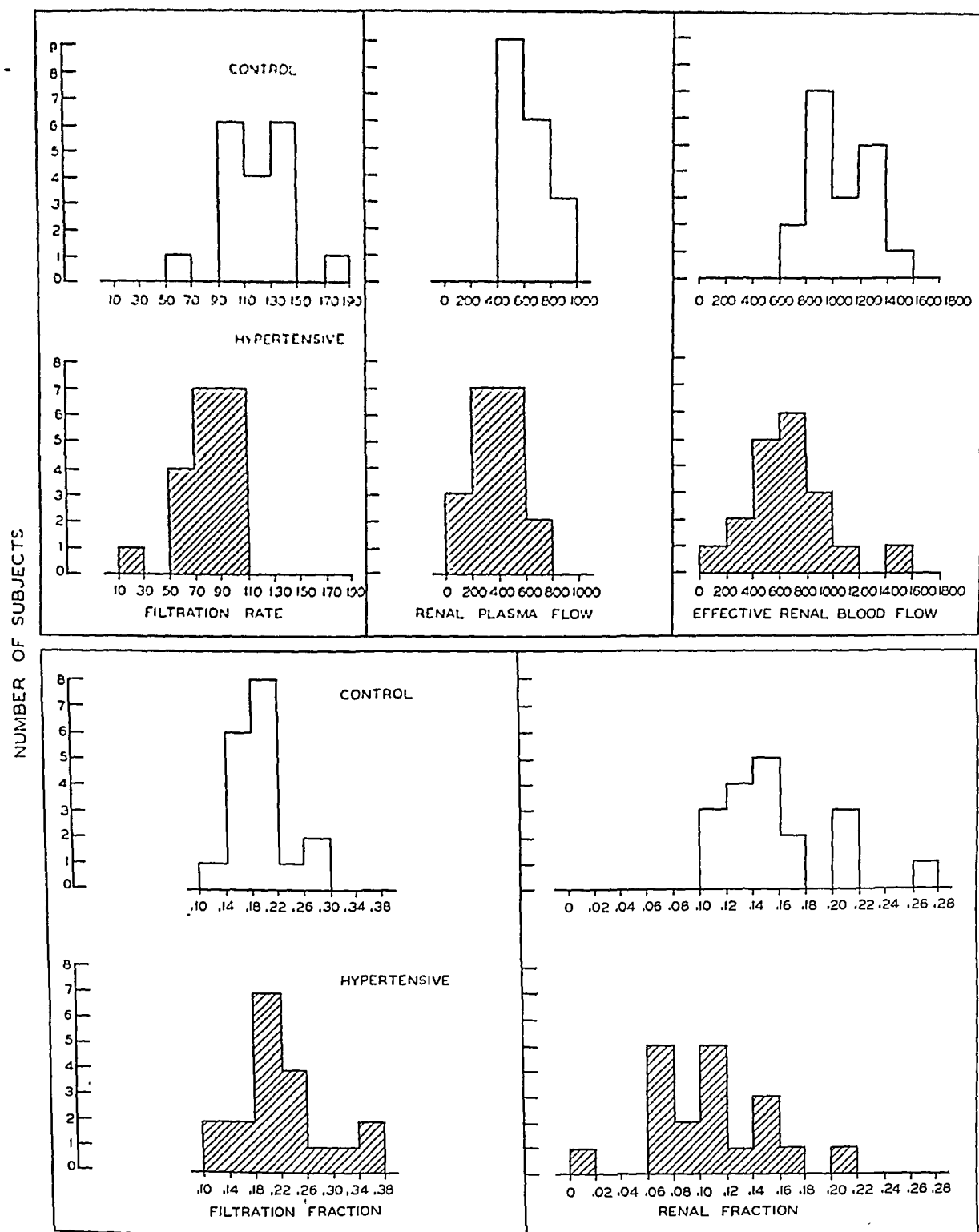


FIG. 2. FREQUENCY DISTRIBUTION OF OBSERVED PHYSIOLOGICAL VARIABLES IN CONTROL AND HYPERTENSIVE SUBJECTS

the first cardiac output determination on each individual. It varies from 0.102 to 0.269, with an over-all average of 0.156 in the control group. The corresponding range for the hypertensives is from 0.014 to 0.207, with an average of 0.106. This average figure of course has little meaning, since the renal blood flow must necessarily decrease with the progressive parenchymal destruction known to be associated with this disease, and if the cardiac output is maintained, the effective renal fraction will decrease correspondingly.⁷

DISCUSSION

As noted in the introductory paragraph, the normal effective renal fraction in the resting post-absorptive condition averages about 0.19 to 0.20 if the normal mean effective renal blood flow reported by Smith (7) is divided by the normal mean cardiac output reported by Cournand, Riley, Breed, Baldwin and Richards (5) and by Stead *et al.* (6).

The lower average of 0.156 for the effective renal fraction in the present control series may be due in part to the influence of moderate anemia on the renal blood flow of some of the subjects. Such a possibility would be suggested from the data of Bradley and Bradley (19). A more important factor, however, is the elevation of the cardiac output. From the data in Table I and from those of Brannon, Merrill, Warren and Stead (20) it would appear that anemia of the moderate degree encountered in these subjects does not, by itself, affect the cardiac output. On

⁷ It should perhaps be emphasized that the term "effective" renal blood flow indicates the volume per minute of blood perfusing functioning excretory tissue (22). On the same basis, "effective" renal fraction is defined as the fraction of the cardiac output which perfuses the functioning renal excretory tissue. If the renal extraction of p-aminohippurate is in the normal range of about 85 to 100 per cent (23, 24), the "effective" renal blood flow and "effective" renal fraction represent 85 to 100 per cent of the "total" renal blood flow and "total" renal fraction. If the extraction is less than normal, the relationship between the "effective" and "total" values will be correspondingly altered. Bradley (23) found that the extraction was normal in 12 of 14 cases of essential hypertension and was reduced only in two patients, both of whom had advanced renal disease with uremia. Judging from Bradley's data, it seems probable that the extraction is moderately or greatly reduced in M.B.I. Furthermore, it is possible that the extraction may be slightly to moderately decreased in M.D., T.V. and M. deL. (see Table II).

the other hand, anxiety, tenseness, annoyance, etc., which frequently complicate this type of investigation, would be expected to raise the cardiac output. This view is supported by the recent studies of Stead *et al.* (6), and of Hickam, Cargill and Golden (21).

It is believed that our control and hypertensive groups are reasonably comparable as regards anemia and anxiety, in so far as may be inferred from the hematocrit, metabolic rate and heart rate. Since the average cardiac output is the same in both groups, it is apparent that the decreased average effective renal fraction in the hypertensive group is attributable to the diminished effective renal blood flow known to accompany this disease.

SUMMARY

Results of simultaneously determined cardiac output by the direct Fick method and renal clearances are presented for 18 nonhypertensive and for 19 hypertensive subjects in the post-absorptive period at rest.

The cardiac indices of control and hypertensive subjects are within the same range, the average for each group being 4.07 and 3.97 liters, respectively.

The average effective renal fraction of the control subjects (0.156, range 0.102 to 0.269) is lower than that calculated from non-simultaneous determinations reported in the literature. This is believed to be a consequence of an increase in average cardiac output associated with the multiple manipulations of simultaneous study, and, to a lesser extent, of the moderate anemia of some of the subjects which may have led to some reduction in the effective renal blood flow.

An additional reduction in the effective renal fraction in the hypertensive subjects (average 0.106, range 0.014 to 0.207) results from the functional ischemia and progressive destruction of renal parenchyma characteristic of the disease, and in advanced hypertensive disease the effective renal fraction may be less than 0.020.

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USE OF BLOOD DONORS WITH POSITIVE SEROLOGIC TESTS FOR SYPHILIS—WITH A NOTE ON THE DISAPPEARANCE OF PASSIVELY TRANSFERRED REAGIN

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Transfusion syphilis is not a serious problem with present methods of blood transfusion. In The Johns Hopkins Hospital there were 12 recognized instances of transfusion syphilis before 1939 (1). Since the establishment of the blood bank in that year, no such cases originating in this hospital have been recognized. With serologically tested blood available at all times, transfusion syphilis is no longer caused by the use of untested blood in conditions of dire emergency.

Of equal importance is undoubtedly the effect of refrigerator storage. With the advent of mass transfusions (6,000 to 7,000 donors are bled each year in The Johns Hopkins Hospital blood bank), and the employment of nurse technicians to bleed the donors, physical examination of donors has become impractical. Obviously, some donors must have given blood at a time when they had lesions of primary syphilis. It is accepted that some 30 per cent of such individuals are seronegative. It is from just such donors that 25 per cent of the cases of transfusion syphilis originated in the past (2). It can hardly be mere chance that we have observed no cases of transfusion syphilis since 1939. Refrigerator storage must be the responsible factor. In 1941 Turner and Discker (3) found that *Treponema pallidum* seeded in rabbit blood kept under conditions simulating those in the blood bank of The Johns Hopkins Hospital failed to give rise to infection when injected into rabbits after storage periods of 48 hours or longer. Bloch (4), simultaneously performing almost parallel experiments, found some persistence of infectivity up to 72 hours but none at 96 hours. Since most bank blood is three to four days old or older when used for blood transfusion and the experimental inoculations employed were much heavier than would be required to reproduce clinical conditions, it would appear that the absence of transfusion syphilis

since the establishment of the blood bank is at least partly attributable to refrigerator storage.

The possible infectivity of plasma has also been studied. Kolmer (5) found inoculated liquid plasma infectious after one and three hours of storage at 4–6° C. but not after 24, 48, or 72 hours. When serum inoculated with treponemes is frozen and desiccated, Turner, Bauer, and Kluth (6) found that under their experimental conditions infectivity of the spirochetes was completely destroyed. When inoculated plasma is frozen and held at –20° C. for 48 hours or longer, Ravitch and Chambers (7) found that the material is not infectious.

In civilian blood banks in areas where the incidence of syphilis in the population is high, as in Baltimore, large numbers of donors are rejected because of a history of syphilis or of anti-syphilitic treatment. Other donors are bled but their blood discarded because of a positive serologic test for syphilis.¹ In 1946, 1.2 per cent of white donors, 13.3 per cent of Negro donors, and 6.5 per cent of all donors—a total of 487 donors—at The Johns Hopkins Hospital, were found to have a positive STS and their blood discarded.

The evidence given above appears to be sufficient to indicate that with the use of plasma in any form (since it is necessarily held for bacteriologic culture for one week before release) there is no danger of transfusion syphilis. The importance of this in a military emergency is obvious. As far as concerns the danger of transmission of syphilis, no STS need be performed and no donor need be rejected in any plasma program. There remains only the problem of whether the passive transfer of reagin produces a positive STS in the recipient. To clarify this phase of the problem, plasma of known reagin titer was injected into individuals with a negative STS.

¹ Hereafter this is abbreviated to STS.

Less direct studies of the fate of passively acquired reagin of syphilis have been made by Fildes (8), Cruickshank (9), Dunham (10), and others (11). They observed that reagin was often passively transferred from the blood stream of women with positive STS to the circulation of their new-born infants. The majority of such infants, although not infected with *Treponema pallidum*, had positive STS at birth. Repeated quantitative STS in these infants showed a definite decline in the titer within seven days. The reactions were usually negative within 30 days, although in an occasional infant the passively transferred antibody could be detected for as long as 90 days. These observations represent passive transfer occurring in nature, analogous to the artificial transfusion of reagin-containing plasma into individuals with a negative STS. Recent studies by the technique of introduction of isotopic nitrogen into antibodies against type-specific pneumococci (12) show that the half-life of such an antibody molecule is approximately two weeks. There is no evidence of continued synthesis of passively transferred antibody.

METHOD

The subjects were all patients on the surgical service of The Johns Hopkins Hospital. At first only patients with known malignant disease were used, but as the results began to show the rapid return of the STS to negative, other types of patients were included. Sixteen patients with negative STS were studied.

All bottles of blood from donors with a positive STS were put aside in the refrigerator. As they accumulated, the plasma was drawn off and pooled, eight to ten donors contributing to each pool. The period of storage before centrifugation of blood and separation of plasma varied from one to ten days. The STS was titrated for each unit of plasma entering the pool and for each pool. The plasma pools were at once divided into units of 500 cc. and then frozen at -10° to -20° C. At intervals of two weeks to two months the frozen plasma was thawed in a water bath at 37° C. and 500 cc. administered intravenously to selected patients. The titer of the STS of each plasma unit after thawing was identical with the original titer. Specimens of blood were drawn from each recipient immediately before the plasma injection and at 24-hour intervals thereafter until the STS by all methods had become negative. In a few recipients several blood samples were taken during the first 24 hours.

Standard quantitative serologic tests were performed with the Mazzini technique (13), the Eagle flocculation (13), the Venereal Disease Research Laboratory Cardiolipin flocculation (14), and the Eagle Wassermann tech-

TABLE I
Serologic response in 16 patients with negative serologic tests for syphilis given 500 ml. of plasma with positive serologic tests for syphilis

Patient		Plasma Injected					Serologic titers in patients 24 hours after injection of plasma				Day after injection on which all serol. tests were negative
No.	Weight	Lot No.	Serologic titers				Comple- ment fixation	Flocculation			
			Comple- ment fixation	Flocculation				Flocculation			
				Eagle	Eagle	Mazzini	Cardiolipin	Eagle	Eagle	Mazzini	
1	55	2	16	8	8	8	4	1	1	2	12
2	66	2					1	D*	D	1	10
3	82	2					D	1	D	D	6
4	39	4					D	D	D	1	4
5	55	4	24	4	4	8	D	1	D	2	19
6	64	4					D	D	D	1	19
7	65	4					D	1	1	2	9
8	42	8					D	1	—	1	14
9	54	8	32	16	16	32	D	1	—	1	20
10	65	8					D	1	—	2	5
11	68	8					D	2	—	2	5
12	59	9					D	D	—	2	12
13	104	9	32	32	32	64	2	2	—	4	9
14	80	10					D	2	—	16	8
15	46	10					4	8	—	32	19
16	60	10					12	16	8	32	19
							4	16	32	32	10

* D = doubtful.

nique (13). Sera were examined within 24 hours after they were drawn. In addition a portion of each serum was frozen at -10°C . In this way all of the specimens drawn from a given patient could subsequently be tested simultaneously after the last specimen had been drawn. Since the results obtained on retesting were practically identical with the original determinations, the results reported here are those obtained with fresh sera. The methods of reporting used by the respective authors of the tests were followed in determining the titers.

RESULTS

1. *Serologic titers of plasma pools.* The serologic titers of the five pools of plasma used in these studies are presented in Table I. In the preparation of pools No. 2 and No. 4 any plasma was used if the corresponding serum had a titer of 1 dilution unit or higher with the Eagle flocculation test. In the pooling of Nos. 8, 9, and 10, plasma was taken only from donors with a titer of 4 dilution units or higher with the Eagle flocculation test. As a result of this the serologic titers in these latter pools were higher. The titers of the pools of plasma varied from 4 to 128 dilution units with the various tests employed.

2. *Serologic titers in sera of recipients within the first 24 hours after the plasma infusion.* Blood specimens drawn from each of the 16 recipients within the first 24 hours after infusion gave positive or doubtful reactions in all cases. The indi-

vidual serologic titers varied from 1 to 32 dilution units in the various tests used (Table I).

3. *Progressive fall in serologic titer in relation to time.* In each case studied the quantitative serologic tests for syphilis gave the maximal response on the first day after infusion. On successive days after transfusion the serologic titers gradually fell in all cases until all serologic tests became negative. There was a rapid fall in titer within the first seven days. Fifteen of the 16 patients had serologic titers of 1 dilution unit or less on the seventh day. The sera of all the recipients became negative within nine to 19 days after injection, with minor fluctuations between tests (Table II). The fall in serologic titer of two of these patients is illustrated in Figures 1 and 2.²

4. *Later serologic follow-up of patients.* None of the patients developed clinical evidence of transfusion syphilis and all of those who could be followed for three to six months maintained their negative STS. Of the 16 patients to whom plasma was given, ten returned for examinations three to six months later and were found to have negative STS at that time. Four of the group had died of their original neoplastic processes, and two could not be found for follow-up examinations.

In reviewing the records of these patients it was found that all of them received penicillin at the time of their surgical procedures. The total dosages of penicillin ranged from 10,000,000 to 50,000,000 Oxford units over periods of seven to 20 days. So that, inadvertently, these patients received adequate treatment for incubating primary syphilis (15). Since under present clinical practice the vast majority of patients sick enough to require transfusions also receive penicillin, the

TABLE II

Duration of positive serologic test for syphilis after injection—comparison of the several serologic tests

Patient No.	Day after injection on which STS became negative				
	Comple- ment fixation	Flocculation			All tests
		Eagle	Eagle	Mazzini	
1	5	7	8	12	12
2	4	4	4	10	10
3	4	4	3	6	6
4	2	2	2	4	4
5	3	8	3	19	19
6	5	5	2	19	19
7	5	9	5	9	9
8	14	3	—	4	14
9	12	15	—	20	20
10	2	5	—	5	5
11	2	5	—	5	5
12	2	12	—	12	12
13	2	9	—	9	9
14	5	9	—	8	8
15	13	19	9	16	19
16	10	10	7	10	10

² While this study was in progress we had the opportunity to observe the effect of a transfusion of STS positive whole blood, erroneously administered. The donor's STS was positive in a titer of 16 dilution units (Eagle). The donor's blood had been stored for ten days at 5°C . The recipient had a negative STS on admission to the hospital. She was a pregnant woman, at term, transfused for secondary anemia. Six days after the transfusion with 500 cc. of STS positive blood her STS was positive (Eagle 2 units, VDRL Cardiolipin 4 units). Nine days after the transfusion all serologic tests were negative. Two days after the transfusion the patient was delivered of a normal infant. The baby's STS was negative when first determined on the ninth day of life.

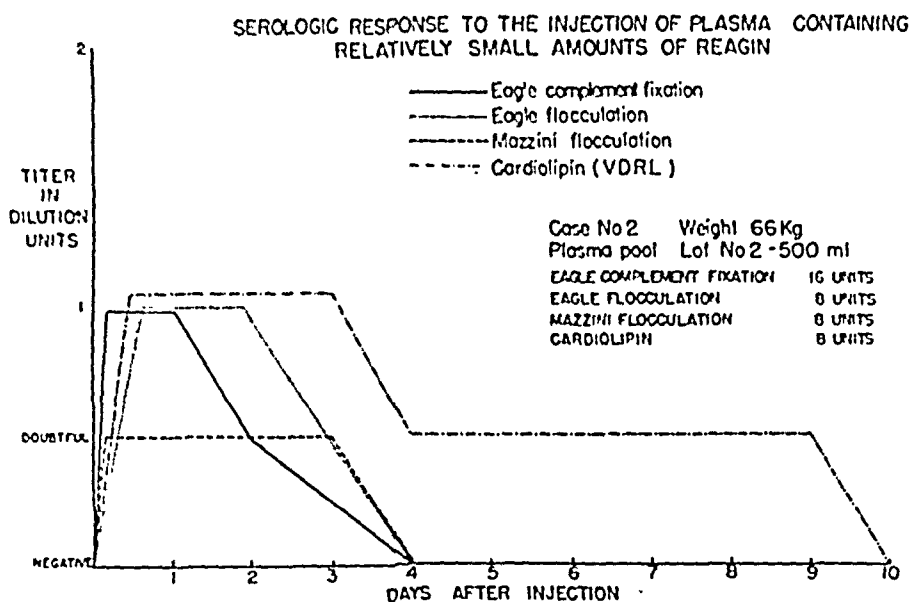


FIG. 1. AFTER INJECTION OF 500 CC. OF PLASMA OF LOW TITER STS, THE RECIPIENT'S IMMEDIATE STS IS POSITIVE IN LOW TITER, AND AT TEN DAYS THE STS IS NEGATIVE BY ALL TESTS

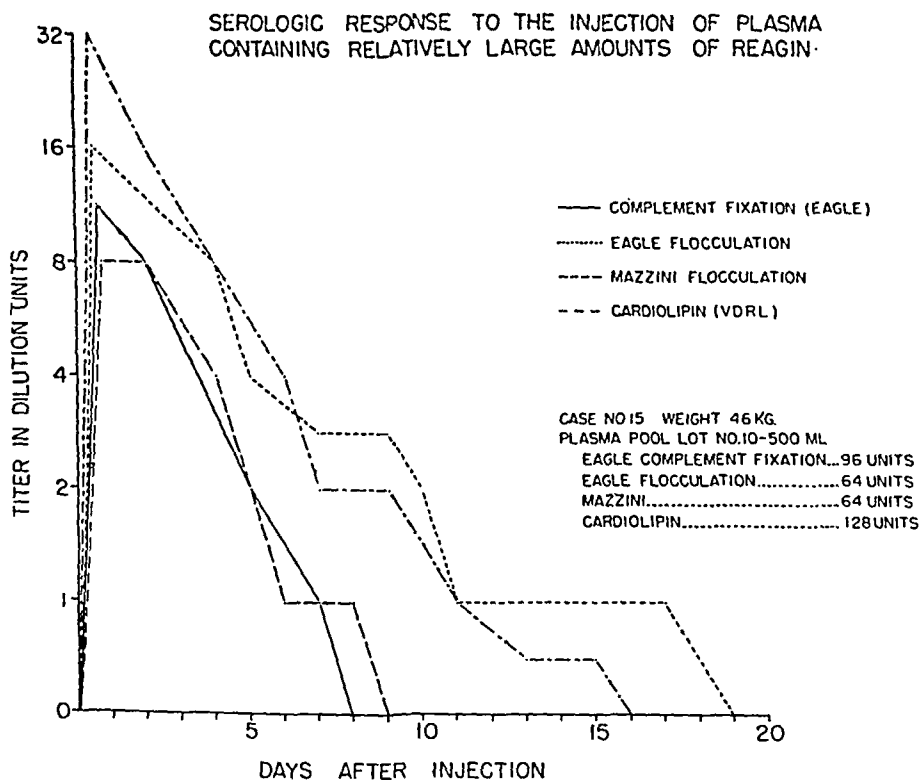


FIG. 2. AFTER INJECTION OF 500 CC. OF PLASMA OF HIGH TITER STS, THE RECIPIENT'S IMMEDIATE STS IS POSITIVE IN HIGH TITER, AND IT REQUIRES 19 DAYS FOR THE STS TO RETURN TO NEGATIVE BY ALL TESTS

likelihood of the occurrence of transfusion syphilis is even further diminished.

DISCUSSION

The weights of the patients varied from 39 to 104 kg. (Table I). If one uses the figure of 45 cc. of plasma volume per kilogram of body weight (16), then the computed plasma volumes for these 16 patients ranged from 1,755 to 4,680 cc. To these initial volumes there was added 500 cc. of plasma containing reagin. It can be calculated that the antibody was diluted *in vivo* approximately one-fifth to one-tenth of its original titer. In the actual tests of the recipients' sera on the day following injection it was observed that one-fourth to $\frac{1}{32}$ of the original titer was found in the recipients' sera. It would appear, within the range of error involved in the computation and in the titration of the sera, that the antibody titer of the recipient after 24 hours represented the dilution of the injected reagin in the blood volume of the recipient.

All of the plasma used in this study was taken from blood of donors known to have a positive STS and preferably of high titer. Yet, even under these extreme experimental conditions, the positive STS in the recipient lasted less than three weeks in the longest instance and only four days in the shortest. If the STS positive blood normally discarded by the blood bank were converted to plasma together with all the STS negative blood usually processed into plasma, no pool would have more than one or two STS positive specimens in it. The resultant pool of plasma would have a very low titer of reagin and the duration of the persistence of the passively transferred positive STS, if any were detectable at all, would be correspondingly brief. In all probability no more than a doubtful reaction would be produced in the recipient.

It is experimentally proved that syphilis will not be transmitted by plasma desiccated or frozen under blood bank conditions. Liquid plasma stored at 4-6° C. one day or more is equally safe. We have now shown that the positive STS passively acquired by the recipient of a transfusion with STS positive plasma is of short duration. The blood of donors with syphilis in any stage should, therefore, be acceptable for use in the plasma program of any institution or organiza-

tion, in which plasma is desiccated, frozen, or refrigerated under the conditions noted above.

SUMMARY

1. Patients transfused with plasma from donors with a positive serologic test for syphilis will acquire a positive STS.
2. The initial titer of the acquired STS represents the dilution in the recipient's blood volume of the reagin contained in the injected plasma.
3. In all of 16 recipients the acquired positive STS had reverted to negative by the 20th day or before.
4. The blood of donors with syphilis should be acceptable for use in any blood bank with a plasma program, provided the material has been frozen, dried from the frozen state, or stored at least four days in the refrigerator.

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HUMAN PROTHROMBIN: QUANTITATIVE STUDIES ON THE PLASMA LABILE FACTOR AND THE RESTORATIVE EFFECTS OF NORMAL, HYPOFIBRINOGENEMIC, AND HEMOPHILIC PLASMA ON THE PROTHROMBIN OF STORED PLASMA¹

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In 1943 Quick (1) reported on the multiple nature of plasma prothrombin. On the basis of his data, which have been repeatedly confirmed (2-4), he concluded that prothrombin consists of two essential factors combined with calcium. The two components, "prothrombin A" and "prothrombin B," had different properties. The former was labile in oxalated or citrated plasma stored at refrigerator temperatures, not adsorbable by aluminum hydroxide, fairly heat stable, and not reduced in dicumarolized blood. Its deterioration was said to account for the high prothrombin time of stored plasma. "Prothrombin B," on the other hand, remained intact in refrigerated plasma, was removed by aluminum hydroxide, was labile at 60° C. for 15 minutes, and was decreased by the administration of dicumarol.

More recently other entities were described which affect the conversion of prothrombin to thrombin by thromboplastin plus calcium (5-7). These are apparently distinct from both "prothrombin B" and the factor which deteriorates in stored plasma. The term "prothrombin A" is now reserved by Quick (5) for one of these substances, and the term "labile factor" for the moiety which is found wanting in stored plasma. Normal prothrombin activity, as determined by the one-stage method, requires the presence of adequate amounts of all these substances.

Loomis and Seegers (4) found that the prothrombin activity of aged plasma could be restored remarkably by the addition of purified fibrinogen. Accordingly, they concluded that deterioration of fibrinogen accounts for lengthening of the prothrombin time in stored plasma and that "reactive

fibrinogen" is necessary for prothrombin activity by the one-stage technique. This conclusion is not in accord with those of Munro and Munro (8) and Honorato and Quick (9). The last authors (9) demonstrated that purified human fibrinogen was incapable of reactivating the prothrombin of stored human plasma. Furthermore, it has also been shown that plasma defibrinated by thrombin could reactivate the prothrombin (9, 10).

The availability of afibrinogenemic plasma from a subject with spontaneous fibrinopenia³ permitted decisive resolution of this conflict. It is the purpose of this report to present data supporting the evidence for the existence of a labile factor which is distinct from fibrinogen and whose deterioration as plasma ages accounts for the increasing prothrombin time. Also included are additional observations on the prothrombin activity of stored plasma and on the restorative effects of hemophilic plasma.

METHODS

Prothrombin activity was determined by the Quick method (11, page 312) with the following modification (12): Commercial (Difco) rabbit brain was used as the source of thromboplastin. Into each of many prothrombin-time tubes were pipetted 0.1-cc. aliquots of a uniform batch of freshly prepared thromboplastin, which were then stored at -10° C. until prothrombin determinations were made. Although thromboplastin thus kept frozen in ready-to-use units maintains full potency for eight months, new thromboplastin preparations were made every two weeks. The control prothrombin value, determined on normal plasma with each fresh batch, served as the control for the two-week period.

In some experiments the prothrombin time was determined on whole plasma unaltered or mixed with afibrinogenemic or hemophilic plasma. In others, the plasma or plasma mixtures were diluted with normal plasma ren-

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dered essentially free of prothrombin by prior adsorption with barium sulfate according to the technique of Rosenfield and Tuft (13). The prothrombin time of the diluted mixture was then determined. By comparing the values thus obtained with those of normal plasma diluted serially in the same manner actual prothrombin activities in per cent of normal were calculated.

Fibrinogen⁴ was measured by the method outlined by Quick (11, page 323). To 1.0 cc. of oxalated or citrated plasma were added 30 cc. of H₂O, 2.5 cc. of 0.02 M CaCl₂, and two drops of a saline solution containing 50 units of thrombin (Topical thrombin, Parke-Davis) per cc. After incubation at 37° C. for 15 minutes, the clot was wound on a glass rod, washed with physiological saline, hydrolyzed with sodium hydroxide and its tyrosine content determined. Since it is almost impossible to remove completely the fibrin clotted in stored plasma by winding on a glass rod, a technique was devised whereby the fibrin was filtered through glass wool, washed and then hydrolyzed in the usual manner. The procedure together with observations on changes in plasma fibrinogen during storage will be reported in detail in a subsequent communication (14).

Oxalated (1 cc. of 0.1 M sodium oxalate to 9 cc. of blood) or citrated (1 cc. of 2.5 per cent sodium citrate to 9 cc. of blood) normal blood was stored at 4-5° C. until its prothrombin time became prolonged. Sterile precautions were used and specimens were cultured to exclude bacterial contamination. Aliquots were then mixed with varying amounts of fresh normal, fresh or freshly frozen fibrinopenic, fresh hemophilic plasmas, or with fresh barium sulfated⁵ plasma, and prothrombin time determined.

RESULTS

Effect of BaSO₄ adsorption on plasma clotting constituents: The prothrombin time of BaSO₄ plasma exceeds 180 seconds, indicating a prothrombin activity of less than 1 per cent of normal. The question arises whether BaSO₄ removes important clotting constituents other than prothrombin. Although plasma so treated loses 100 mgm. of protein per 100 cc. (13) its fibrinogen concentration and antihemophilic potency were found unchanged. Also (see below) it is fully capable of rectifying the delayed prothrombin time of stored plasma. It is noteworthy that BaSO₄ removes far less prothrombin from citrated than from oxalated plasma.

Prothrombin activity of aged plasma and restorative ability of BaSO₄ plasma: The activity of normal oxalated plasma stored at 4-5° C. decreases to about 3 per cent of normal within three weeks

(Table I). Citrated plasma, on the other hand, deteriorates more slowly. These observations agree with those of Quick (15). At body temperature the loss is much more rapid although here, too, citrated plasma is more stable. Full restoration of the prothrombin activity to stored refrigerated plasma is induced not only by the admixture of fresh normal citrated or oxalated plasma (as found by others [1, 8]) but also by BaSO₄ plasma (Tables I, II). Substantial restoration to incubated plasma can similarly be obtained (Table I). Indeed, mixtures of fresh BaSO₄ plasma with oxalated plasma aged for more than 14 days at 4-5° C. exhibit much more prothrombin activity than the original plasma before, or within the first week

TABLE I

Effect of temperature and anticoagulant on prothrombin activity in stored plasma

1. Pools of oxalated and citrated plasma, subdivided and stored at 4-5° C.*								
Storage	Oxalated				Citrated			
	Undiluted plasma		Diluted 1:10 with fresh BaSO ₄ plasma		Undiluted plasma		Diluted 1:10 with fresh BaSO ₄ plasma†	
	Prothrombin		Prothrombin		Prothrombin		Prothrombin	
days	time sec.	per cent	time sec.	per cent	time sec.	per cent	time sec.	per cent†
0	14.8	42	21.4	118	12.1	100+	18.3	180
3	15.7	32	23.4	100	12.2	100+	21.5	115
7	21.9	11	24.0	96	11.5	100+	22.0	110
10	43.6	4	19.1	160	14.8	41	21.4	115
17	60.0	2.8	17.6	215	15.9	30	17.8	210
32	50.0	3.2	19.1	160	21.7	11.3	17.2	220
35	58.0	3.0	18.8	172	—	—	—	—
45	60.0	2.8	20.2	130	—	—	—	—
49	—	—	—	—	61	2.8	18.2	194
54	—	—	—	—	38	4.2	21.2	118
2. Oxalated and citrated plasma (from same donor) kept at 37° C.								
hours	time sec.	per cent	time sec.	per cent	time sec.	per cent	time sec.	per cent
0	14.0	50	28.0	75	11.9	100+	27.3	80
1	14.2	45	29.4	68	13.2	72	28.2	74
3	16.2	25	29.7	67	13.1	80	30.1	65
6.5	17.1	23	31.9	58	13.4	65	32.2	58
24	29.7	6.8	33.2	55	18.8	17	33.6	50

* Culture of a representative sample of both oxalated and citrated plasma, performed on the seventh day of storage, showed no growth. The pH of both on this day was 8.52 and 8.06 respectively.

† Corrected for the 1:10 dilution with the barium sulfated plasma.

⁴ The term "fibrinogen" in this paper refers to that plasma protein component which is clotted by the addition of thrombin.

⁵ Hereafter referred to as "BaSO₄ plasma."

TABLE II

Restorative effect of normal or prothrombin-free plasma on lost prothrombin activity of stored plasma (4-5° C.)

Plasma mixture	Prothrombin		
	Time	Concentration (per cent of normal*)	
		Expected	Found
Fresh cit. plasma	sec.		
Stored (45 days) oxal. plasma	12.0	100	100
One part cit. plasma plus 1 part stored oxal. plasma	116.0	—	1.3
	12.7	51	80-100
Fresh oxal. plasma	13.8	100	100
BaSO ₄ oxal. plasma	more than 15 min.	0	0
One part fresh oxal. plus 9 parts BaSO ₄ plasma	25.9	10	9
One part fresh oxal. plus 19 parts BaSO ₄ plasma	40.2	5	5
Stored (7 days) oxal. plasma	28.7	—	7
One part stored plus 9 parts BaSO ₄ fresh plasma	21.6	0.7	11.5
One part stored plus 1 part fresh plus 18 parts BaSO ₄ fresh plasma	23.3	5.3	10
One part fresh plus 9 parts stored plasma	21.0	16.3	12
One part fresh plus 2 parts stored plasma	15.9	38	30
Fresh oxal. plasma	14.5	100	100
BaSO ₄ oxal. plasma	more than 15 min.	0	0
One part fresh plus 9 parts BaSO ₄ plasma	25.9	10	9
Stored (10 days) oxal. plasma	41.5	—	4.3
One part stored plus 9 parts BaSO ₄ plasma	23.9	0.4	10
One part fresh plus 9 parts stored plasma	21.8	13.9	11
Two parts fresh plus 8 parts stored plasma	17.6	23.4	21
Three parts fresh plus 7 parts stored plasma	16.4	33	25
Four parts fresh plus 6 parts stored plasma	15.0	42.6	40-80
Five parts fresh plus 5 parts stored plasma	14.2	52	100

* Calculated on the basis of fresh normal plasma having 100 per cent prothrombin activity.

of storage despite the fact that the prothrombin time of the undiluted plasma has become very prolonged.

Prothrombin activity of congenital afibrinogenemic plasma and its restorative properties on prothrombin activity of stored plasma: As is evident in Table III, afibrinogenemic plasma from a patient with congenital afibrinogenemia can fully reactivate the prothrombin of stored plasma. This is all the more significant since a mixture of equal parts of both contains half the fibrinogen concentration of the stored plasma alone. Fibrinogen, therefore, is not the factor whose deterioration accounts for the retarded prothrombin time of stored plasma.

The afibrinogenemic plasma failed to clot following the addition of thromboplastin plus calcium. According to the orthodox one-stage method of determining prothrombin, this would be interpreted as indicating zero prothrombin. When, however, measured by the BaSO₄ plasma dilution technique its prothrombin activity was normal.

Relation between prothrombin activity and plasma fibrinogen concentration: The prothrombin time of mixtures of fresh normal plasma with congenital afibrinogenemic plasma or plasma defibrinogenated by the addition of thrombin does not change significantly until the fibrinogen drops to approximately 100 mgm. per cent (Table IV). Below this the prothrombin time increases until at 17-25 mgm. per cent the apparent prothrombin activity is about 11 per cent of normal. Furthermore, at this point the clot becomes translucent and coagulation is not abrupt. In three weeks old plasma (4-5° C.), on the other hand, prothrombin activity is about 3 per cent of normal but coagulation is sharp, the clot is opaque and fibrinogen concentration is well above 100 mgm. per cent (14). These observations also support the interpretation that deterioration of fibrinogen does not explain the elevated prothrombin time of stored plasma.

Prothrombin and prothrombin restorative activity of hypofibrinogenemic blood from a patient

TABLE III

The effect of the addition of plasma from patient with congenital afibrinogenemia on the prothrombin activity of stored plasma

Plasma mixture (Parts)						Prothrombin	
Afibrino- genemic* (Citrate)	Saline	Stored†	Fresh normal	Barium sulfated normal	Barium sulfated stored‡	Time	Concent.
—	—	—	1	9	—	sec.	per cent of normal§
—	—	1	—	—	—	29.1	70
—	—	1	—	9	—	68	2.2
1	—	—	—	—	—	23	103
—	—	1	1	—	—	α	
1	—	1	—	—	—	12.6	100±
—	—	1	—	—	—	12.5	100±
—	—	9	1	—	—	19.2	158
1	—	9	—	—	—	18.0	200
—	—	—	2	—	8	29.2	35
1	—	—	1	—	8	27.7	33.5
—	1	—	1	—	8	53.5	30
1	—	—	—	9	—	26.5	83

* This plasma was citrated. All the others were oxalated. 1 cc. of this plasma to which was added 30 cc. of physiological saline, 2.5 cc. of 0.02 M CaCl₂ solution and two drops of thrombin solution (400 units per cc.) showed no clot within four hours.

† Stored 19 days at 4–5° C. Cultured and found sterile.

‡ The plasma which was stored for 19 days was then treated with barium sulfate to remove prothrombin activity.

§ Corrected for dilution.

with liver disease: In a patient with fibrinopenia⁶ and liver disease prothrombin activity as determined by the usual procedure was about 10 per cent of normal. When, however, prothrombin was measured on a mixture of the subject's plasma with prothrombin-free BaSO₄ plasma, higher but still subnormal prothrombin values were obtained. Clearly the BaSO₄ plasma provided a clotting agent, either fibrinogen, or other non-prothrombin substances, which was inadequate in the fibrinopenic plasma and which is necessary for full prothrombin activity. That normal prothrombin activity was not found in the mixture was to be expected in view of the extensive liver disease which this patient had (carcinoma of pancreas with diffuse involvement of the liver).

As with the congenital afibrinogenemic plasma, this fibrinopenic plasma lowered the elevated prothrombin time of stored plasma although not to the same degree as did normal plasma.

The effect of defibrination of plasma by thrombin on its ability to reactivate the prothrombin of stored plasma: We were able to confirm the ob-

⁶ Plasma fibrinogen concentration was 53 mgm. per 100 cc.

TABLE IV

Relationship between plasma fibrinogen concentration and prothrombin activity

Experiment 1. Afibrinogenemic plasma from subject with congenital fibrinopenia							
Plasma mixture (parts)		Fibrinogen concent. mgm. per cent*	Prothrombin		Properties of clot observed		
Afibrino- genemic	Normal		Time	Concent.	Volume	Opacity	Sharpness of endpoint
0	1.0	250	sec.	per cent			
1.0	3.0	188	13.0	100	normal	opaque	sharp
1.0	1.0	125	12.5	100	normal	opaque	sharp
2.3	1.0	76	12.8	100	normal	opaque	sharp
4.0	1.0	50	14.7	40	small	translucent	sharp
9.0	1.0	25	16.5	25	small	very translucent	sharp
			22.2	11	very small	very translucent	not sharp
Experiment 2. Normal plasma defibrinated by addition of thrombin (5 units to 1 cc. of plasma)							
0	1.0	171	13.7	100	normal	—	sharp
3	7.0	120	13.2	100	normal	—	sharp
5	5.0	86	13.3	100	normal	—	sharp
6	4.0	68	14.2	46	small	—	sharp
7	3.0	51	14.4	43	small	—	sharp
8	2.0	34	16.5	25	small	—	sharp
9	1.0	17	22.8	10	small	—	sharp
19	1.0	8.5	45.0	4	very small	—	not sharp

* Assuming 250 mgm. per cent fibrinogen in the normal plasma. (This value was obtained on the same subject one month later.) The fibrinopenic plasma was shown to be completely devoid of fibrinogen but normal in prothrombin content.

servations of others (10, 16) that plasma defibrinated by thrombin can partially reactivate the prothrombin of stored plasma. That it cannot do so fully is perhaps due to some inactivation of labile factor by the prolonged incubation (one hour) required for complete defibrination and inactivation of whatever excesses of thrombin may have been present. Another possibility is partial removal of the labile factor by the fibrin clot.

The effect of heat defibrinogenation of plasma on its ability to restore prothrombin activity to stored plasma: Human plasma heated at 55½–56° C. for two minutes retains some prothrombin (27–40 per cent) but has lost its ability to reactivate the prothrombin of stored plasma. Although such treatment removes fibrinogen, the loss in prothrombin restorative ability was not referable to defibrination since adequate amounts of fibrinogen were provided in the mixture of heat-treated with stored plasma.

Can hemophilic plasma restore prothrombin activity to stored plasma? The admixture of hemophilic plasma to stored plasma resulted in complete reactivation of its prothrombin activity. The coagulation defect of hemophilic blood cannot therefore be attributed to deficiency in labile factor, which is in accord with the normal prothrombin activity in this disease.

Amount of labile factor necessary to reactivate the prothrombin of stored plasma: The reactiva-

TABLE V
Relation between prothrombin activity and ratio of fresh to stored plasma in mixture of both

Plasma* mixture		Prothrombin	
Normal	Stored (7 days, 4–5° C.)	Time	Concentration
per cent	per cent	sec.	per cent of normal
100	0	13.8	100±
0	100	28.7	7.3
10	90	21.0	12.0
29	71	17.0	23.0
33	67	15.9	40±
50	50	14.7	—
	(10 days, 4–5° C.)		
100	0	14.5	100±
0	100	41.5	4.3
10	90	21.8	11.2
20	80	17.6	21.3
30	70	16.4	25.0
40	60	15.0	40±
50	50	14.2	—

* Oxalated plasma throughout.

TABLE VI
Relation between concentrations of labile factor and plasma prothrombin activity

Plasma mixture (Parts)					Prothrombin	
Stored* incu- bated	Fresh† normal	Incu- bated barium sulfated	Mixt. cont. theoretical amounts of labile factor	"Proth. B"‡	Time	Activity
			per cent of normal	per cent of normal	sec.	per cent of normal
0	1	—	100	96	13.0	100
0.2	0.8	—	80	106	13.1	100
0.3	0.7	—	70	103	13.1	100
0.4	0.6	—	60	101	13.7	60–100
0.5	0.5	—	50	99	15.0	39
0.6	0.4	—	40	92	16.5	25
0.7	0.3	—	30	96	20.6	12.3
0.8	0.2	—	20	91	26.3	8.4
0.9	0.1	—	10	99	58.3	2.9
1.0	0	—	0	95	>3 min.	<1
	Normal barium sulfated	Stored incub. barium sulfated				
0.5	0.5	—	50	49	18.2	19
0.5	0.4	0.1	40	49	20.4	12.8
0.5	0.3	0.2	30	49	24.6	9.4
0.5	0.2	0.3	20	49	28.3	7.5
0.5	0.1	0.4	10	49	51.0	3.2

* Stored 39 days at 4–5° C. Incubated for 24 hours at 37° C.

† The normal plasma contained 336 mgm. fibrinogen per 100 cc.

‡ Determined by adding 0.1 cc. of the mixture to 0.9 cc. of fresh normal barium sulfated plasma according to the technique of Rosenfield and Tuft (13).

tion of stored plasma prothrombin by progressively increasing admixtures of fresh plasma is shown in Table V. As the per cent of fresh plasma is increased, the prothrombin time decreases, but does not become normal until the mixture contains at least 50 per cent fresh plasma. It is noteworthy that up to 40 per cent the increment in prothrombin activity induced by the fresh plasma is practically equivalent to the absolute amount of prothrombin activity added with the fresh plasma. Above 40 per cent, relatively normal prothrombin times are approached which unfortunately cannot be accurately translated into prothrombin concentrations since they are in the least accurate range of the curve which relates prothrombin time to prothrombin concentrations.

Additional experiments (Table VI) were done in which increasing amounts of fresh plasma were added to stored plasma which had been incubated for 24 hours prior to the experiment. In this way, the labile factor of the stored plasma was completely destroyed whereas its "prothrombin B" was only partially inactivated. Similar mixtures were made using BaSO₄ plasma which was also incubated.

A sharp drop in prothrombin activity ensues when the amount of labile factor is decreased below 60 per cent of normal. Of interest, also, is the fact that a given deficiency of labile factor has a greater effect on prothrombin activity when the "prothrombin B" concentration is 50 per cent of normal than when it is normal.

It should be mentioned that the stored incubated plasma (Table VI) had no demonstrable fibrinogen. Nevertheless, the data are still valid, since amounts of fibrinogen requisite for substantial prothrombin activity are certainly provided by those mixtures containing at least 30 per cent of normal, or normal BaSO_4 plasma (see Table IV).

DISCUSSION

That afibrinogenemic plasma from a patient with spontaneous fibrinopenia is fully capable of reactivating the prothrombin of stored plasma is conclusive evidence that the restorative factor provided by fresh plasma is not fibrinogen. This is further substantiated by the restorative properties of normal plasma rendered fibrinogen-free by thrombin. Although restoration under the latter conditions was only partial, any reactivation by fibrinogen-free plasma excludes fibrinogen as being the restoring agent. The discordant results obtained with fibrinogen by Loomis and Seegers (4) are probably referable to contamination of their fibrinogen with labile factor.

Thus far the existence of labile factor is predicated upon observations that the prothrombin time increases as plasma ages although its prothrombin concentration does not change, and that this can be rectified by the admixture of whole fresh plasma, or plasma devoid of fibrinogen and prothrombin. This does not necessarily imply that it exists as an entity, albeit independent of these coagulation substances. It is conceivable that the above properties are referable to a particular configuration in the molecule of a plasma constituent distinct from fibrinogen and prothrombin, and essential for prothrombin activity as determined by the orthodox one-stage technique.

The prolonged prothrombin time of fibrinopenic plasma, of stored plasma, and of the plasma from recently described patients whose clotting defects were not referable to true hypoprothrombinemia (5, 17) demand reevaluation of the method for measuring prothrombin by the one-stage proce-

dure. Its inadequacies derive from the fact that in all these instances prolonged prothrombin times are observed despite normal prothrombin (B) concentrations. It is also pertinent to point out that the use of physiological saline as a plasma diluent in order to bring the prothrombin time into a range more suitable for accurate conversion into prothrombin content may aggravate these inadequacies by diluting, simultaneously with the prothrombin, other coagulation factors whose concentrations may already be critical. Clearly, these weaknesses of the one-stage technique can best be obviated by diluting the test plasma with plasma which has been rendered prothrombin-free in some manner and which still retains all other clotting factors. As far as is known, BaSO_4 plasma fulfills these criteria, although we are cognizant of the statement by Owren (6) that BaSO_4 removes some clotting "factor V" from plasma. (The exact amount of BaSO_4 required to do this is not indicated.)

Little is known regarding the exact quantitative relationships between labile factor concentration and apparent prothrombin activity. Munro and Munro (18) claim that there is practically no change in prothrombin time over a wide proportion of labile factor and "prothrombin B" and that a deficiency of one component can be partially compensated for by an excess of the other (3). This is not entirely in accord with our finding.

Thus far no case is recorded in which a deficiency of labile factor has been observed. It appears that reducing it below 50 per cent of normal can result in substantial prolongation of the prothrombin time. The experiments on our patient with extensive liver disease suggest that this subject was somewhat deficient not only in prothrombin and fibrinogen, but also in labile factor since his plasma could not completely reactivate stored plasma prothrombin. That this cannot be referable to low fibrinogen concentration is clear from the quantitative relationships between fibrinogen concentration and prothrombin time (Table IV). It is possible that with a more practical technique for labile factor determination such as that of Stefanini and Quick (16) more patients will be found with coagulation defects related to insufficiency of this entity.

Our observations on the heat sensitivity of labile factor are not in accord with those of Quick

(1), who claimed that it is fairly heat stable. In our experience, the labile factor is quite rapidly inactivated at body temperature, and completely destroyed at $55\frac{1}{2}$ – 56° C. within two minutes, which agrees with the finding of Honorato (10). Under these conditions "prothrombin B" is more stable.

The reason why BaSO_4 does not remove all the prothrombin from citrated (in contrast to oxalated) plasma is obscure. In this connection it also is noteworthy that aluminum hydroxide adsorbs prothrombin less effectively from heparinized than from oxalated plasma (1).

The enhanced prothrombin activity of plasma stored for more than two weeks (when determined with BaSO_4 plasma as diluent) is striking and cannot at present be explained. Honorato (10) similarly found that a mixture of stored plasma with fresh plasma treated with $\text{Al}(\text{OH})_3$ shows more prothrombin activity than the fresh plasma alone. He attributes this to qualitative changes in fibrinogen consequent to storage which renders it more easily coagulable. Experiments which will be presented in a subsequent communication (14) indicate that this cannot be the explanation since the fibrinogen of stored plasma becomes progressively less coagulable while at the same time the prothrombin activity, when measured by the BaSO_4 plasma dilution technique, increases. It is unlikely that more prothrombin can develop as plasma ages. Furthermore, no such increase in prothrombin is found by actual isolation or when the two-stage technique for prothrombin determination is used (19). It is possible that a naturally occurring clotting inhibitor (20) disappears as plasma is aged.

CONCLUSIONS

1. The prothrombin activity of stored plasma as determined by the one-stage technique can be fully reactivated by the admixture of fresh normal plasma, congenitally afibrinogenemic plasma, BaSO_4 plasma (prothrombin-free) or hemophilic plasma. The restorative factor (labile factor) is not fibrinogen.

2. The prothrombin time of plasma can be greatly influenced by alterations in the concentrations of (a) prothrombin, (b) labile factor and (c) fibrinogen.

3. Prothrombin-free BaSO_4 plasma has a normal amount of labile factor, fibrinogen, and anti-hemophilic activity. Its use as a diluent in measuring prothrombin by the one-stage technique is advantageous since in this way these non-prothrombin variables are adequately controlled.

4. Plasma stored for more than two weeks shows enhanced prothrombin activity when measured by the BaSO_4 plasma dilution technique. The explanation of this phenomenon is obscure.

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A STUDY OF THE RATE OF DISAPPEARANCE OF A DIGITALIS GLYCOSIDE (LANATOSIDE C.) FROM THE BLOOD OF MAN¹

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INTRODUCTION

Exact quantitative determinations of either digitalis or its derivatives in the blood of patients or animals receiving these drugs have never been accomplished. The prime difficulty of course has been the inability to detect relatively minute amounts of digitalis or allied cardiotonic drugs in body fluids. Because of the absence of such quantitative data, very little is known about the actual fate in the body of either ingested or injected digitalis substances.

In previous studies (1, 2) we were able to detect by means of the embryonic duck heart preparation extremely minute amounts of a digitalis glycoside (Lanatoside C.) in both Tyrode's solution and in human blood. Moreover, the quantity of glycoside detectable (less than a microgram per cubic centimeter of blood) by this means suggested the possibility that the blood of patients receiving Lanatoside C. might be assayed for its glycoside content.

Accordingly, blood samples of ten patients who had received intravenously a therapeutic quantity of Lanatoside C. were assayed. It was found possible not only to detect in a quantitative fashion the presence of glycoside in such samples but also to measure the rate of the drug's disappearance from the blood stream.

METHODS

A. Quantitative detection of Lanatoside C. in human serum (*in vitro*)

In a previous study (2) it was found that the embryonic duck heart exhibited a "digitalis effect" (the occurrence of A-V block, missing beats or marked retardation of beating) when immersed in human serum containing 0.10 μ g. or more of Lanatoside C. per cc. Furthermore, it was observed that the time necessary for

the occurrence of the "digitalis effect" varied inversely with the amount of glycoside present in the serum samples. Finally, it was observed over a period of 20 months that no temporal variation occurred in the sensitivity of these embryonic hearts to a given concentration of Lanatoside C. These last findings offered the possibility of a method for the quantitative detection of glycoside.

Accordingly, different but known amounts of Lanatoside C. were added (0.10 to 1.0 μ g. per cc. of serum) to samples of human serum and the various samples were refrigerated for 24 hours. Then five embryonic duck hearts were added to each sample and the individual times elapsing before occurrence of the "digitalis effect" were observed. This procedure was repeated until over 20 hearts had been exposed and observed in at least four different samples of serum containing the same concentration of glycoside. From these data, it was possible to construct a table whereby the quantity of Lanatoside C. in any serum sample could be determined by observation of the average time taken for five or more embryonic hearts to exhibit a typical "digitalis effect."

B. Quantitative detection of Lanatoside C. in the patients' sera

Serum samples were obtained from ten patients (seven of whom were suffering from either right or left congestive heart failure) who had not received any type of digitalis therapy within three months. To 1 cc. samples of each patient's serum, 0.10, 0.15 and 0.20 μ g, respectively, of Lanatoside C. were added. These samples together with one not containing glycoside were refrigerated for 24 hours and then each was tested on five embryonic hearts. The embryonic hearts were found to beat normally for at least 80 minutes in the control serum samples of each patient. Furthermore, the times elapsing for the occurrence of "digitalis effect" in hearts immersed in the various glycoside-serum samples corresponded in every case to the times previously observed for the same concentrations of Lanatoside C. as listed in the standard table. It was considered, therefore, that the serum of each of these patients was satisfactory for the quantitation by the duck heart method of possible Lanatoside C. content.

Approximately 1.6 mgm. of Lanatoside C. were given by vein over a period of three minutes to each of the ten patients. Blood samples (10 cc.) were obtained from the vein of the opposite arm immediately after the injection of the glycoside and again after 7½, 15, 22½ and 30

¹ Aided by grants from the Life Insurance Medical Research Fund, the U. S. Public Health Service, and The Sandoz Chemical Works.

minutes. In several instances, samples also were taken at later intervals.

The blood samples were allowed to clot, the serum was removed and stored in the ice box for 24 hours. Six embryonic hearts then were added to two portions of each serum sample and the individual times elapsing before the occurrence of "digitalis effect" in each heart was noted exactly as described previously (1, 2). The time periods were averaged and compared to the standard table correlating the time elapsing before occurrence of "digitalis effect" in the embryonic hearts with the concentration of Lanatoside C. per cc. of serum. If a sample did not cause a "digitalis effect" within a period of 80 minutes, it was assumed, because of the previous study with known concentrations, that the sample contained less than 0.1 μ g. of glycoside per cc. of serum. Therefore 0.1 μ g. of the drug was added to 1 cc. of the same sample and tested again. If then the average time elapsing before occurrence of "digitalis effect" was the same as that occurring in samples known to contain 0.1 μ g. per cc. it was assumed that the unknown sample contained less than 0.05 μ g. per cc. If, on the other hand, such a sample caused the appearance of the "digitalis effect" in a time period equal to or shorter than that previously observed in samples containing 0.15 μ g. per cc. it was concluded that the unknown sample contained at least 0.05 μ g. per cc. In this manner, 0.05 μ g. of Lanatoside C. per cc. of serum could be detected.

RESULTS

A. The quantitative detection of Lanatoside C. in human serum (in vitro)

As Table I demonstrates, serum samples containing concentrations of Lanatoside C. differing for the most part by only 0.1 μ g. per cc. could be

quantitatively differentiated from each other by observation of the time of occurrence of "digitalis effect." The standard error of the mean of the values observed at any concentration was small enough to allow such quantitation except when concentrations of 0.8 μ g. or more of glycoside per cc. of serum were present. These latter concentrations, however, were never reached in the blood of patients receiving therapeutic amounts of Lanatoside C.

B. The quantitative detection of Lanatoside C. in patients' sera after its intravenous injection

As Table II indicates, the serum of each of the patients contained a detectable amount of Lanatoside C. immediately after the intravenous administration of 1.6 mgm. The average concentration of the glycoside was 0.25 μ g. per cc. of serum (Range: 0.20 to 0.40). No significant difference was found in the serum concentration of the drug in patients with or without congestive failure.

The initial concentration of the glycoside, however, was not maintained for when nine serum samples obtained 7.5 minutes after administration of the drug were assayed, it was found that the average concentration of Lanatoside C. had fallen to 0.10 μ g. per cc. of serum (Range: 0.05 to 0.15). At the end of 15 minutes, no glycoside could be detected in the sera of two of the ten patients. The sera of the remaining patients contained only 0.05 to 0.15 μ g. per cc. At the end of 22.5 minutes, only two of eight patients tested still contained a detectable amount of Lanatoside C. in their blood (0.05 to 0.10 μ g. per cc.). At the end of 30 minutes, no Lanatoside C. (i.e., less than 0.05 μ g. per cc.) could be found in the sera of nine of the ten patients. At the end of an hour, no glycoside was detected in the sera of the four patients tested. Finally the sera of two patients who exhibited signs of digitalis intoxication at the time the 24-hour sample was taken, 24 hours after injection did not contain a detectable amount of Lanatoside C.

DISCUSSION

The quantitative detection of Lanatoside C. in the blood of a patient or animal treated with this drug allows the future possibility of studying the fate of other digitalis glycosides in the human or

TABLE I

Relationship between concentration of Lanatoside C. and time of occurrence of "digitalis effect"

Concent. of glycoside	Number of hearts	Time of occurrence, "digitalis effect"	
		Average	Standard error of mean
μ g./cc.		min.	min.
0.05	20	No effect	—
0.10	26	62	1.2
0.15	29	56	1.3
0.20	33	43	1.2
0.30	22	32	2.0
0.40	21	26	1.4
0.50	26	22	1.8
0.60	20	17	1.4
0.70	20	15	0.5
0.80	22	13	0.9
0.90	20	11	0.9
1.00	20	11	0.8

TABLE II

The disappearance of Lanatoside C. from the serum of patients after its intravenous administration
Concentration of Lanatoside C. in serum after its injection

Subject	Cong. heart failure?	Immediately		7.5 Min.		15 Min.		22.5 Min.		30 Min.		60 Min.		24 Hours	
		TDE*	µg./cc.	TDE	µg./cc.	TDE	µg./cc.	TDE	µg./cc.	TDE	µg./cc.	TDE	µg./cc.	TDE	µg./cc.
J. G.	Yes	37	0.25	55†	0.05	62†	ND‡	61†	ND	64†	ND	64†	ND	60†	ND
R. S.	Yes	32	0.30	57	0.15	58	0.15	62	0.10	62†	ND	64†	ND	—	—
R. R.	Yes	36	0.25	54	0.15	52†	0.05	55†	0.05	60†	ND	62†	ND	—	—
J. M.	Yes	37	0.25	58	0.15	50†	0.05	61†	ND	66†	ND	—	—	—	—
E. I.	Yes	49	0.20	—	—	54†	0.05	—	—	64†	ND	—	—	—	—
J. J.	Yes	45	0.20	54	0.15	52†	0.05	65†	ND	63†	ND	—	—	—	—
R. Z.	Yes	44	0.20	62	0.10	56†	0.05	63†	ND	62†	ND	—	—	—	—
U. K.	No	43	0.20	60	0.10	66†	ND	67†	ND	65†	ND	—	—	—	—
C. C.	No	25	0.40	62	0.10	61	0.10	—	—	56†	0.05	—	—	62†	ND
J. S.	No	34	0.30	63	0.10	54†	0.05	60†	ND	67†	ND	68†	ND	—	—
Average		38	0.25		0.10										

* TDE equals average time of occurrence of "digitalis effect" in five embryonic hearts.

† 0.1 µg. of Lanatoside C. added to each cc. of serum sample.

‡ ND indicates that no Lanatoside C. could be detected in serum sample.

animal body. Little is known at present about this matter.

The present study has resulted not only in a quantitative estimation of the actual amount of Lanatoside C. in the blood of a patient immediately after the intravenous administration of the drug but even more important, has allowed us to determine its rate of disappearance from the blood stream.

In a previous study (2) it was found that when this particular glycoside was added to whole blood, little or none was adsorbed or contained in the blood cells. Therefore, the rapid disappearance of Lanatoside C. from the serum of our patients (usually over 80 per cent within 22.5 minutes after administration) could not be explained by its entrance into or adsorption by blood cells. Nor could the rapid disappearance be due to inactivation of the Lanatoside C. by serum, which was found in the previous study to be unable to do so (2). Also, in view of the fact that the plasma proteins were not able to adsorb this particular glycoside (2), it seems most likely that the rapid disappearance of Lanatoside C. from the blood stream was due to its actual escape therefrom by the simple process of diffusion.

It is perhaps worthy of note that the rate of disappearance of the drug from the blood stream did not appear to be affected by the presence or absence of congestive heart failure. It is interesting too that two patients experienced symptoms of

digitalis intoxication 24 hours after administration of Lanatoside C., at which time none of it could be detected in their blood.

SUMMARY

1. The quantitative detection of small amounts of Lanatoside C. in human serum was accomplished.
2. The rate of disappearance of this glycoside from the blood of ten human subjects after its intravenous administration was observed.
3. The probable reason for the rapid disappearance of Lanatoside C. from the blood stream of the experimental subjects was discussed.

ACKNOWLEDGMENTS

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THE RENAL REGULATION OF ACID-BASE BALANCE IN MAN. III. THE REABSORPTION AND EXCRETION OF BICARBONATE¹

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In the normal individual the concentration of bicarbonate in the extracellular fluid is maintained within limits of 24 and 28 mM per liter despite wide variations in the intake of acid and base forming foodstuffs. Stabilization of level depends on continuously operative renal mechanisms, for although carbonic acid is continuously available, no large reserve depots of base exist upon which the body may draw when circulating stores are depleted, or to which the body may consign an excess in times of abundance. The renal problem of regulating the concentration of bicarbonate may be outlined in the following terms: 1, conservation of those stores which normally enter the renal tubules in the glomerular filtrate; 2, excretion of any excess present in the body; and 3, conversion of the salts of fixed acids into bicarbonate to replenish depleted body stores.

These aspects of regulation have been studied in normal human subjects at plasma bicarbonate levels ranging from 13 to 39 mM per liter. It has been observed at plasma concentrations below 24 mM per liter that essentially all of the filtered bicarbonate is reabsorbed; negligible quantities are lost in the urine. Above 28 mM per liter a relatively constant quantity is reabsorbed, amounting on an average to 2.8 mM per 100 ml. of glomerular filtrate; any excess present in the filtrate is excreted. These properties of the reabsorptive mechanism would in themselves assure stabilization of plasma level were there no continuous drain on bicarbonate stores for purposes of neutralizing fixed metabolic acid. Because such a drain exists in the individual maintained on the usual acid ash diet, the renal tubules must split neutral salts of the glomerular filtrate, restoring the fixed base to the body as bicarbonate, and eliminating the unwanted anions either as free titratable acid or in combination with ammonia.

Within limits these processes of replenishment proceed at rates proportional to the degree of depletion of the alkali reserves. The data upon which these findings are based are reported below.

METHODS

Fourteen experiments were performed on three healthy male subjects in the post-absorptive state. Adequate hydration was assured by the ingestion of two to four glasses of water at the start of each experiment. In some experiments plasma bicarbonate was reduced by the ingestion of 5 to 20 gms. of ammonium chloride on the preceding day. In all experiments plasma bicarbonate was elevated by the intravenous infusion of 1.5 to 3.0 per cent sodium bicarbonate at rates of 5 to 13 ml. per min. The thiosulfate clearance was used as a measure of glomerular filtration rate (1, 2).

Urine was collected with special precautions to avoid loss of carbon dioxide. One subject, who could empty the bladder completely of even small volumes of urine, voided into a funnel connected with a rubber tube dipping beneath oil. A close fit between funnel and glans minimized exposure of the urine to air. The other two subjects were catheterized and the catheter was extended under oil by attaching to it a length of rubber tubing. The urine was permitted to drain continuously through the catheter and at the end of the collection period the bladder was emptied by voluntary effort and abdominal compression. The customary procedure of washing out the bladder and introducing air at the end of each urine collection was avoided because of the error it causes in the determination of urinary carbon dioxide tension. Although more constant clearances were obtained in the subject who voided spontaneously, the similarity of carbon dioxide tensions in urine samples collected by the two methods, as well as the stability of tension for some six to eight hours after collection, indicated that both methods adequately protected against carbon dioxide loss. Arterialized venous blood was drawn and analyzed by methods described in a previous communication (3).

RESULTS

The reabsorption and excretion of bicarbonate as a function of plasma concentration

Two illustrative experiments on a single subject are presented in Table I; the first two clear-

¹ Aided by grants from the United States Public Health Service and the John and Mary R. Markle Foundation.

TABLE I
The relationship between the reabsorption and excretion of bicarbonate and chloride and the plasma concentration of bicarbonate

Urine flow ml./min.	Plasma				Urine				Bicarbonate				Chloride				Sum
	BHCO ₃	pCO ₂	pH	Chlo- ride	BHCO ₃	pCO ₂	pH	Chlo- ride	Filtered	Excreted	Reabsorbed	mM/100 ml. glomerular filtrate	Filtered	Excreted	Reabsorbed	mM/100 ml. glomerular filtrate	
	mM/L	mm.Hg		mM/L	mM/L	mm.Hg		mM/L	mM/min.	mM/min.	mM/min.	mM/100 ml. glomerular filtrate	mM/min.	mM/min.	mM/min.	mM/100 ml. glomerular filtrate	
Experiment 1. R. F. P.	3.97	100.3	7.27	109.2	0.0147	36.0	5.28	114.4	1.34	0.00006	1.34	1.34	10.95	0.453	10.40	10.37	11.71
	3.80	102.6	7.27	108.6	0.0129	34.6	5.23	109.6	1.39	0.00005	1.39	1.35	11.15	0.415	10.73	10.45	11.80
	3.40	99.1	7.29	108.0	0.0114	32.0	5.21	105.0	1.37	0.00004	1.37	1.38	10.70	0.357	10.34	10.44	11.82
	3.33	101.1	7.28	107.5	0.0113	31.7	5.21	97.0	1.52	0.00004	1.52	1.50	10.86	0.323	10.54	10.41	11.91
	3.53	109.3	7.33	107.0	0.0112	31.4	5.21	87.6	1.74	0.00004	1.74	1.59	11.70	0.308	11.39	10.41	12.00
	3.67	117.0	7.35	106.0	0.0115	31.1	5.23	81.8	1.96	0.00004	1.96	1.67	12.40	0.300	12.10	10.34	12.01
	3.06	108.2	7.38	105.6	0.0121	31.1	5.24	69.4	1.91	0.00004	1.91	1.76	11.44	0.212	11.23	10.38	12.14
	3.00	115.4	7.38	105.2	0.0121	32.0	5.24	60.2	2.12	0.00004	2.12	1.84	12.13	0.181	11.95	10.37	12.21
Experiment 2. R. F. P.	4.73	123.0	7.39	106.8	0.0311	42.4	4.48	59.8	2.51	0.0002	2.51	2.04	13.14	0.282	12.86	10.46	12.50
	4.76	124.0	7.39	107.0	0.0239	35.6	4.44	61.6	2.53	0.0001	2.53	2.04	13.26	0.293	12.97	10.46	12.50
	7.08	126.0	7.43	105.2	1.30	59.5	5.95	54.4	3.06	0.0082	3.05	2.42	13.25	0.384	12.87	10.21	12.63
	4.33	122.0	7.44	104.6	12.2	82.6	6.78	80.6	3.15	0.0530	3.10	2.54	12.77	0.349	12.42	10.18	12.72
	4.94	128.0	7.48	103.8	33.1	84.1	7.21	75.8	3.53	0.164	3.37	2.63	13.30	0.376	12.92	10.10	12.73
	8.46	134.0	7.51	100.8	83.0	90.6	7.58	58.2	4.37	0.702	3.67	2.74	13.51	0.493	13.02	9.72	12.46
	11.10	132.0	7.54	98.8	99.2	90.6	7.64	49.8	4.74	1.10	3.64	2.76	13.05	0.550	12.50	9.48	12.24
	13.30	136.0	7.56	96.8	105.0	97.1	7.65	47.2	5.20	1.40	3.80	2.79	13.17	0.627	12.44	9.14	11.93

ance periods of each experiment constitute control observations made prior to the infusion of bicarbonate. Sufficient ammonium chloride had been ingested on the day preceding Experiment 1 to reduce the plasma bicarbonate concentration to 13.4 mM per liter, pH to 7.27, and partial pressure of carbon dioxide to 30.5 mm. Hg in the initial two periods. The urine was acid in reaction and contained negligible quantities of bicarbonate. The quantity of bicarbonate filtered each minute was calculated by multiplying the plasma concentration, expressed in mM per ml., by the glomerular filtration rate in ml. per min. The quantity excreted was calculated by multiplying the urine concentration, expressed in mM per ml., by the rate of urine flow in ml. per min. The quantity reabsorbed is equal to the difference between the quantities filtered and excreted. It is evident that under conditions of moderately severe acidosis over 99.99 per cent of the filtered bicarbonate was reabsorbed; only a negligible trace was excreted.

Following the control periods of Experiment 1, the plasma concentration of bicarbonate was gradually raised from 13.5 to 18.4 mM per liter by the infusion of bicarbonate, the reaction of the plasma

returning to a normal value, namely pH 7.38. In consequence of the increase in plasma concentration and the increase in filtration rate, the quantity of bicarbonate delivered into the renal tubules rose from 1.34 to 2.12 mM per min. Reabsorption of the filtered bicarbonate was essentially complete throughout this range. Likewise in Experiment 2 at a somewhat higher control level, reabsorption was equally complete. However, as the plasma level was raised to or above 25.8 mM per liter, the renal tubules failed to reabsorb all of the bicarbonate presented to them, and significant quantities appeared in the urine.

It is apparent from the two experiments presented in Table I that the rate of reabsorption of bicarbonate increased in direct proportion to the increase in plasma concentration; *i.e.*, reabsorption increased from 1.34 to 3.80 mM per min. as plasma concentration rose from 13.4 to 38.2 mM per liter. However, there occurred coincidentally an increase in glomerular filtration rate, and since previous work on the dog (3) has shown that the tubular capacity to reabsorb bicarbonate at any given plasma level varies in proportion to filtration rate, the data were calculated in terms of mM reabsorbed per 100 ml. of glomerular filtrate. It is

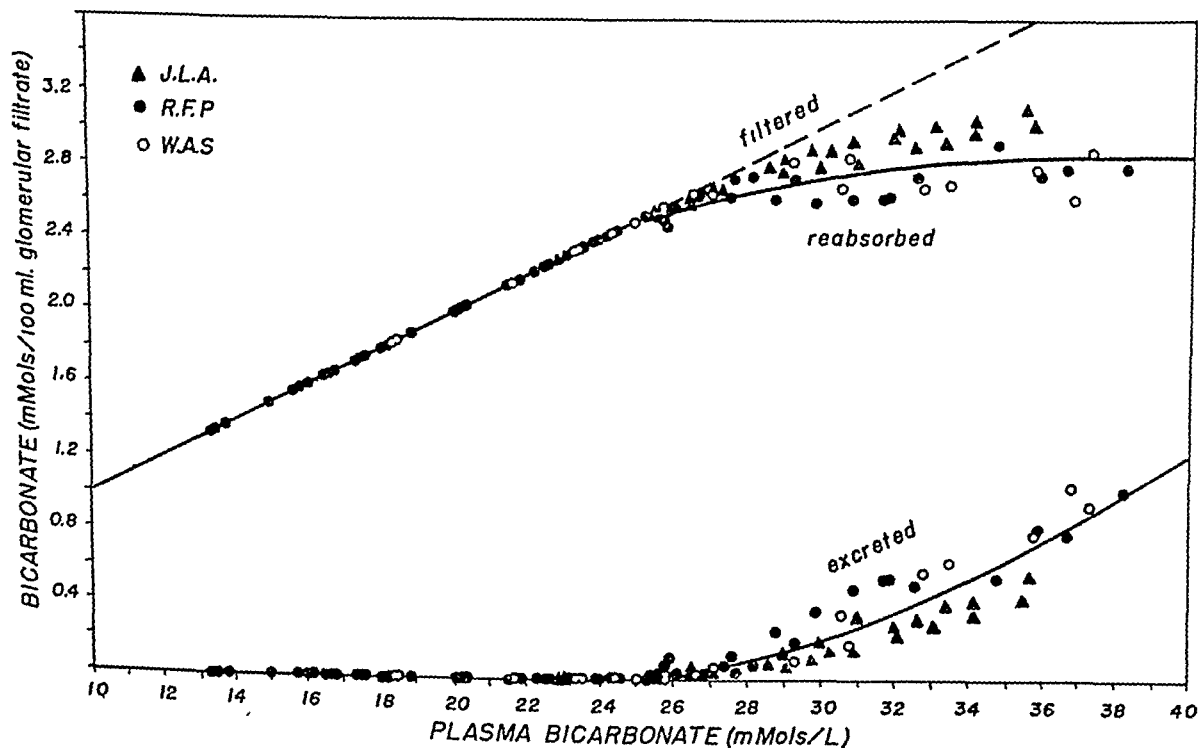


FIG. 1. THE REABSORPTION AND EXCRETION OF BICARBONATE AS A FUNCTION OF PLASMA LEVEL

evident from Table I that bicarbonate reabsorption expressed in this fashion increased with plasma concentration up to a plasma level of 27.6 mM per liter. From 27.6 to 38.2 mM per liter reabsorption was essentially constant, varying only from 2.63 to 2.79 mM per 100 ml. of filtrate.

In Figure 1 are presented the massed data derived from 14 experiments on three subjects. The reabsorption and excretion of bicarbonate, expressed in mM per 100 ml. of glomerular filtrate, are plotted against plasma bicarbonate concentration. It is evident that reabsorption of filtered bicarbonate was essentially complete at plasma levels below 24 mM per liter. Above 28 mM per liter gross excretion occurred, and reabsorption was limited to an average value of 2.8 mM per 100 ml. of glomerular filtrate (varying within limits of

2.6 and 3.0 mM). Thus the renal plasma threshold² for bicarbonate in this series of experiments varied only within narrow limits of 25 to 27 mM per liter. It must be emphasized that this threshold probably holds exactly only under our experimental conditions of polyuria, acutely elevated bicarbonate concentration, and otherwise normal plasma electrolyte pattern.

Interaction in the reabsorption of chloride and bicarbonate

The ingestion of ammonium chloride converts a portion of the circulating store of bicarbonate into chloride, the increase in the latter roughly equaling the decrease in the former. In diarrhea

² Renal threshold is used in the sense of that plasma concentration above which significant excretion occurs.

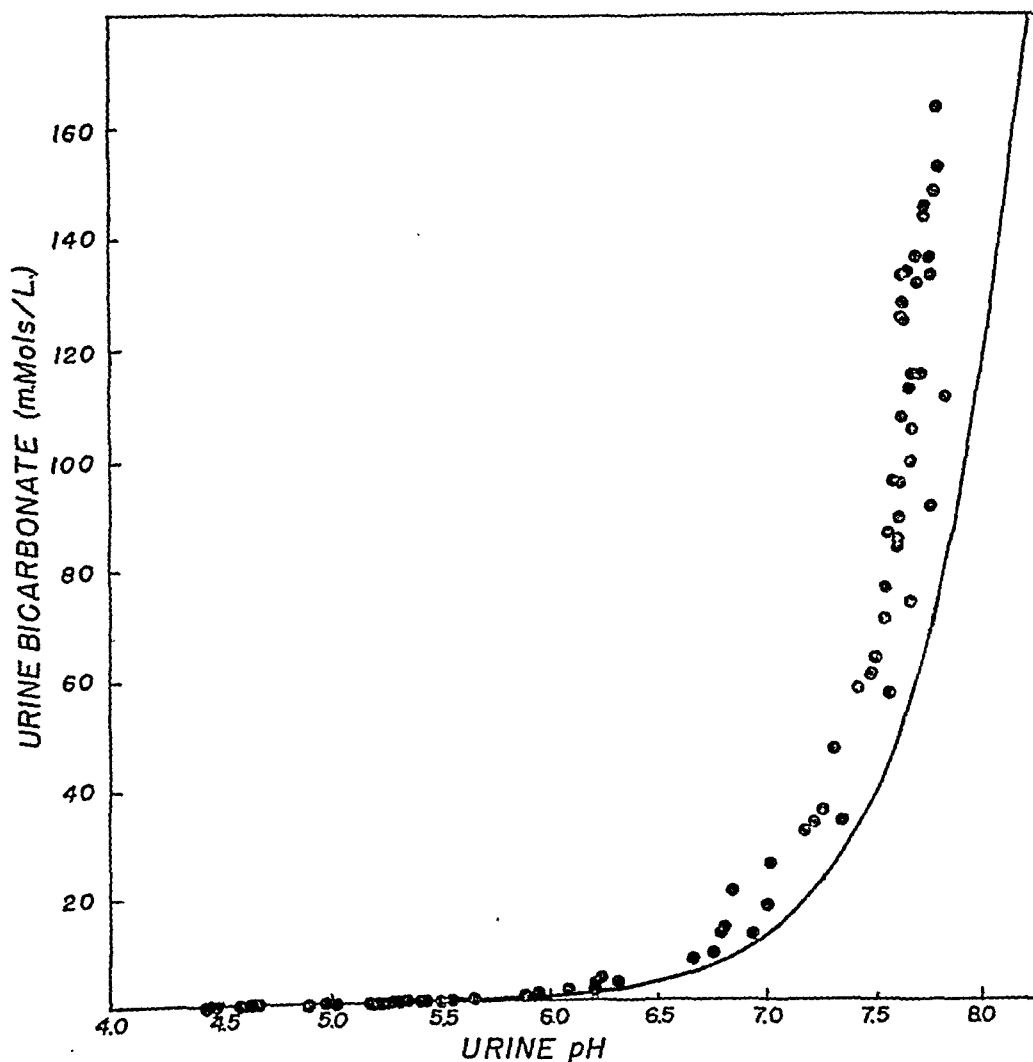


FIG. 2. THE RELATIONSHIP BETWEEN BICARBONATE CONCENTRATION AND pH IN 82 SPECIMENS OF URINE SO COLLECTED AS TO PREVENT LOSS OF CARBON DIOXIDE

The smooth curve was calculated from the Henderson-Hasselbalch equation assuming a constant $p\text{CO}_2$ of 50 mm. Hg.

STUDIES IN DIABETIC ACIDOSIS AND COMA, WITH PARTICULAR EMPHASIS ON THE RETENTION OF ADMINISTERED POTASSIUM

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Abnormally low concentrations of potassium, at times associated with muscular paralysis, have been observed in the serum of diabetic patients while under treatment for acidosis or coma (1-4). Available data indicate that deficits of cell potassium are also present in at least some of these subjects (5). In an attempt to characterize and quantitate the magnitude of these deficits, exchanges of water, electrolytes, carbohydrate, and protein have been investigated in diabetic acidosis and coma prior to, during, and following the administration of potassium salts.

EXPERIMENTAL PROCEDURE AND METHODS

Eight cases in all have been studied. Of these, two were adults and the remaining six juvenile diabetics 16 years of age or younger. On admission all had Kussmaul breathing, a marked reduction of the serum bicarbonate content, hyperglycemia, glycosuria, and ketonuria.

Studies during treatment and recovery were divided into three periods. The first or pre-KCl period extended from admission to the point where the patients had improved sufficiently to be maintained on an oral intake. During this time insulin, 0.9 per cent saline, and, once the blood sugar began to drop, glucose solutions were administered in amounts summarized in Table I. Under this treatment ketosis diminished, overbreathing ceased, hyperglycemia decreased, and mental clarity returned. This interval lasted 12 to 25 hours in the individual subjects. During the second or KCl period, 22 to 37 hours in length, the patients received insulin, 10.0 to 30.0 grams of KCl *per os* or intravenously, whole milk containing added carbohydrate, and water as desired. The third or post-KCl period began after the final dose of KCl and lasted up to 34 hours. During this time the patients received insulin as needed, together with measured amounts of water and milk.

In all subjects concentrations of the whole blood non-protein nitrogen, blood sugar (6-8), and the levels of serum chloride, bicarbonate, sodium, potassium and water were measured at the beginning and end of each period (6, 9-12). Average values for the electrolyte, carbo-

hydrate, and protein content of fresh milk have been used in calculating the intake (13, 14). Urine excreted during each period was analyzed for nitrogen, sodium, potassium, chloride, and glucose (6, 10, 11, 15, 16). Body weight was determined when possible at the start and end of each period.

METHOD OF CALCULATION

Alterations in extracellular fluid volume were calculated from changes in the chloride space, based on the external balance of this anion and corrected for changes in the serum concentrations of chloride as described by Elkinton and co-workers, and by Darrow (17-19). In view of the dehydration known to develop in the course of diabetic acidosis and coma, the initial extracellular volume was assumed for purposes of this calculation to be 15 per cent of the body weight rather than the usual value of 18.7 to 23 per cent found in non-dehydrated human subjects (20). In two instances a pre-treatment weight could not be obtained. A reasonable assumption was therefore made as to its magnitude, based upon subsequent weights and the balance of water. The volume of the chloride space and the concentrations of sodium and potassium in extracellular water, corrected for the Donnan effect, were used in the calculation of increments and decrements of these ions in extracellular fluid. The retention of sodium and of potassium by cells was taken to be the difference between the intake of these cations and the amounts retained in extracellular fluid plus those excreted in urine. In the case of potassium the final values recorded include corrections for the balance of cellular nitrogen, using the K:N ratio of 2.38 m.eq.:1.6 gm. N. In determining the balance of cellular nitrogen changes in the concentration of the nonprotein nitrogen have been taken into account, assuming for this purpose that the nonprotein nitrogen is distributed uniformly through all of the body water, and that 0.65 of the body weight is water. Similar treatment of the sodium data does not, of course, significantly affect the balances of this electrolyte because of the low Na:N ratio in cells. The data on cell base in Table III represent, therefore, balances in excess of the sodium and potassium which move with nitrogen in the anabolism and catabolism of protein.

TABLE I
Intake and output during therapy of diabetic acidosis and coma

Pt.	Period	Intake							Urine output					
		Insulin	H ₂ O	Cl	Na	K	N	CHO	Vol.	Cl	Na	K	N	Glucose
T.W.	I	<i>units</i> 650	<i>ml.</i> 13,335	<i>m. eq.</i> 1,723	<i>m. eq.</i> 1,723	<i>m. eq.</i> 49	<i>gms.</i> 0	<i>gms.</i> 300	<i>ml.</i> 1,675	<i>m. eq.</i> 178	<i>m. eq.</i> 92	<i>m. eq.</i> 37	<i>gms.</i> 7.6	11
	II	135	3,333	629	212	490	12.1	270	1,180	192	85	39	7.3	13
	III	75	2,150	71	57	85	11.8	268	2,300	442	262	90	10.5	27
M.S.	I	170	6,255	726	726	8	0.6	132	1,663	79	38	55	10.9	24
	II	5	3,075	182	38	192	8.0	74	2,190	279	133	83	10.4	0
	III	20	1,025	34	29	42	6.8	43	970	121	92	20	5.8	0
J.K.	I	240	5,240	769	769	0	0	132	3,490	257	265	74	8.6	155
	II	120	5,275	488	69	505	14.2	343	4,050	523	187	208	11.1	65
	III	15	800	20	16	24	3.3	98	315	33	10	16	1.2	14
W.S.	I	154	4,000	615	615	0	0	75	1,100	165	116	53	6.1	26
	II	0	4,350	303	289	322	12.0	155	4,200	718	421	183	4.8	59
	III	58	1,550	51	41	61	8.5	74	2,136	33	207	60	8.4	22
L.D.	I	280	6,570	775	775	0	0	143	4,330	566	473	65	7.1	67
	II	20	5,510	457	170	380	15.9	138	2,800	306	106	36	6.1	5
	III	70	4,525	131	106	158	21.9	190	4,510	409	232	51	11.7	2
R.J.	I	500	6,750	998	1,025	3	8.4	115	1,775	139	106	28	10.0	39
	II	105	4,800	288	16	292	3.8	29	818	133	78	30	9.8	1
	III	50	2,500	16	13	20	2.7	24	1,790	249	146	45	12.7	45
D.C.(a)	I	245	6,876	1,020	1,020	0	0	127	3,630	355	275	105	14.1	70
	II	105	2,550	475	58	490	12.0	105	6,580	990	584	251	21.7	167
	III	57	3,200	105	85	126	17.5	152	2,000	168	16	88	16.6	83
D.C.(b)	I	230	6,925	973	973	0	0	275	4,250	73	164	124	5.4	129
	II	90	6,135	495	90	518	18.5	404	6,710	846	475	233	15.7	268
	III	70	3,175	75	61	91	12.6	185	1,770	72	24	60	7.9	60

RESULTS

A. Alteration in serum water and in extracellular volume

On admission a variable degree of plasma dehydration, as measured by the water content, was present (Table II). The values ranged from 897 to 924 gms. of water per liter of serum in contrast to levels of 922 to 938 gms. per liter ordinarily found in healthy adults (21). There is no means of deciding how much of the deficit in any particular patient should be assigned to losses of body fluid alone, and how much is to be considered secondary to a disappearance of circulating plasma proteins of the type observed during depletion of body water and solutes (22, 23). At the end of the first period of treatment all serum water values were again in the normal range, or actually above it. No consistent pattern is apparent in the fluctuations in serum water during the second and third treatment periods.

The expansion in the chloride space uniformly

observed in each patient at the end of the first treatment period suggests that considerable deficits of extracellular water as well had been incurred prior to admission (Table III). These increments, as calculated from the chloride space, ranged from +1.0 to +9.5 liters. Since subsequent changes in Periods II and III of each study in no instance entirely cancelled this initial expansion it is reasonable to interpret this persistent positive balance of extracellular water as indirect evidence suggestive of extracellular dehydration on admission. On the other hand, it is likely that in at least some of the subjects the extracellular fluid volume had been overexpanded, even though edema did not develop. This appeared to be true in some of the infants with diarrhea treated by Darrow (19).

B. Serum electrolyte concentrations prior to and during therapy

1. *Sodium and chloride:* Despite these deficits of body water it is to be noted that the concen-

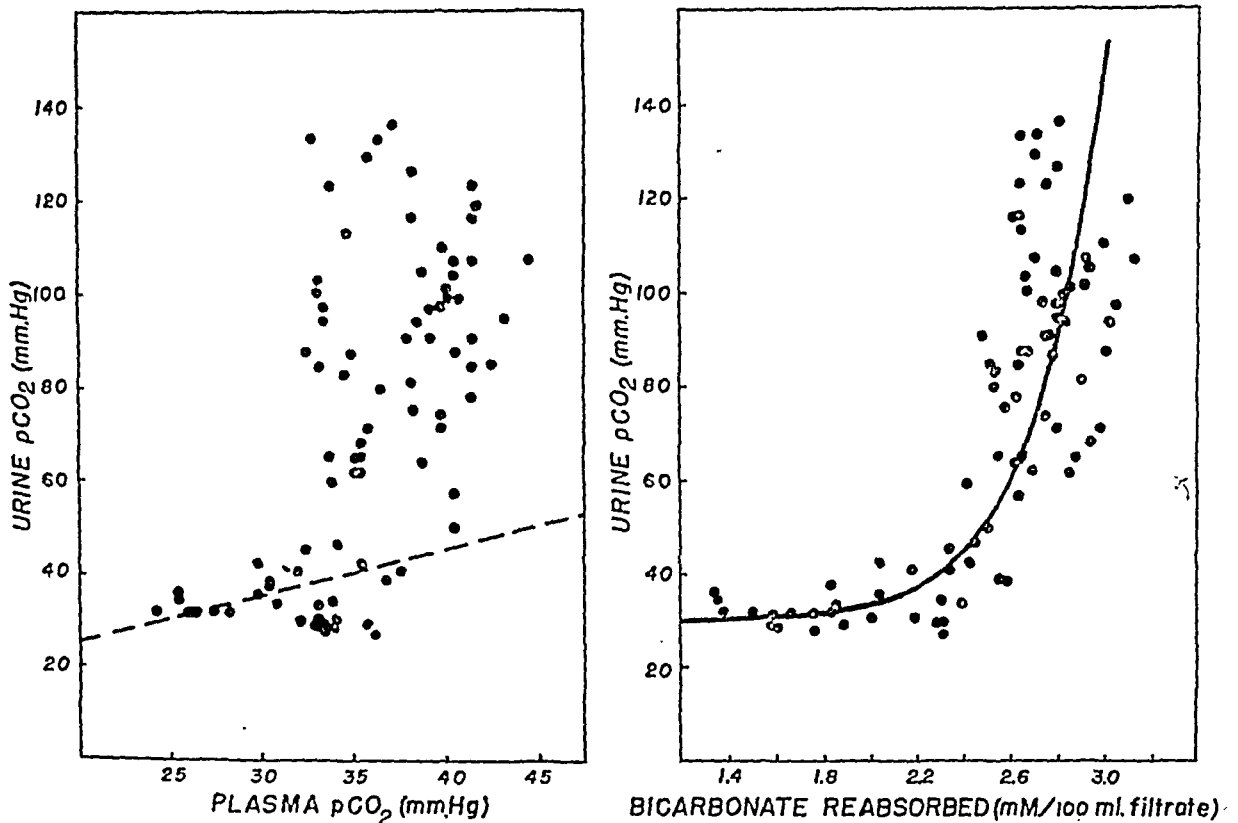


FIG. 3. LEFT: THE RELATIONSHIP BETWEEN THE $p\text{CO}_2$ OF THE URINE AND THE $p\text{CO}_2$ OF THE PLASMA
 RIGHT: THE RELATIONSHIP BETWEEN THE $p\text{CO}_2$ OF THE URINE AND THE REABSORPTION OF BICARBONATE

a similar disturbance of electrolyte pattern results from the loss of alkaline intestinal secretions (4, 5). In contrast in pernicious vomiting, loss of chloride is attended by a compensatory elevation of plasma bicarbonate (4, 6, 7). Thus there exists an inverse relationship between the plasma concentrations of chloride and bicarbonate, whereby the sum of the two is maintained relatively constant. Under conditions of stress, the kidney, failing to maintain the normal anion pattern, at least approximates maintenance of constant total anion (hence cation) concentration. As illustrated in Experiment 2 of Table I, this appears to result from compensatory changes in the quantities of chloride and bicarbonate reabsorbed, when one is presented to the renal tubules in excess. Thus 10.46 mM of chloride were reabsorbed per 100 ml. of glomerular filtrate at a plasma bicarbonate concentration of 20.4 mM per liter. As the bicarbonate level was raised the reabsorption of chloride fell and the excretion rose. The sum of chloride and bicarbonate reabsorbed, however, remained roughly constant.

The acid-base equilibrium of the urine

When an excess of base is liberated in the body in consequence of the metabolism of an alkaline ash diet, it is converted to bicarbonate and excreted in the urine. Gamble (8) and Marshall (9) have shown that the carbon dioxide tension of the urine roughly approximates that of the blood, hence large quantities of base bound as bicarbonate can be eliminated in urine of moderate alkalinity. As much as 220 mM of bicarbonate can be excreted in a liter of urine of pH 8.0. In Figure 2, the concentration of bicarbonate in the urines from our 14 experiments are plotted against urine pH. The reaction of the most acid urine was pH 4.44, that of the most alkaline, pH 7.80. The maximum concentration of bicarbonate was 164 mM per liter, a figure which is relatively low because of the diuretic conditions in our experiments; the minimum concentration was 0.00001 mM per liter. The smooth curve of Figure 2 was calculated from the Henderson-Hasselbalch equation assuming a urine $p\text{CO}_2$ of 50 mm. Hg. It is evident that above

pH 6.5 the reaction of the urine is less alkaline than would be predicted from its content of bicarbonate. This deviation results from the fact, evident in Table I, that urines of high bicarbonate content have a higher $p\text{CO}_2$ than that assumed in calculating the curve. As shown in Figure 3, carbon dioxide tensions as high as 136 mm. Hg were observed in some urines of high bicarbonate content.

In Figure 3 the urinary partial pressure of carbon dioxide was plotted in two ways, on the left, against the simultaneously determined plasma partial pressure, and on the right, against the quantity of bicarbonate reabsorbed per 100 ml. of glomerular filtrate. It is evident from the lefthand graph that the $p\text{CO}_2$ of the urine is with few exceptions equal to or greater than that of the plasma from which it was formed. Those points below the dashed line of equivalence may be low because of inaccuracies introduced by loss of carbon dioxide or because of variations of pK' which would render the calculations inexact. The relationship, however, is less significant than that demonstrated in the righthand graph, which relates urinary $p\text{CO}_2$

and bicarbonate reabsorption. It is evident that at values of reabsorption less than 2.0 mM per 100 ml. of filtrate, $p\text{CO}_2$ was relatively constant, at values greater than this it increased rapidly. For reasons which will be amplified subsequently, it is believed that in the process of distal tubular reabsorption of bicarbonate, carbonic acid is liberated in the tubular urine. Were it possible to plot urine $p\text{CO}_2$ against distal rather than total bicarbonate reabsorption, a more exact correlation might be evident.

The relation between titratable acid and ammonia excretion and alkali reserve

Since the excretion of acid anions in combination with ammonia and as free titratable acid serves to replenish body stores of bicarbonate, the rates of both processes are determined in part by the extent of depletion of the alkali reserve (10). *Base economy*, which serves as a measure of the effective restoration of this reserve, is determined as the sum of the rates of ammonia and titratable acid excretion minus the rate of bicarbonate excretion (11). The contribution of each of these compo-

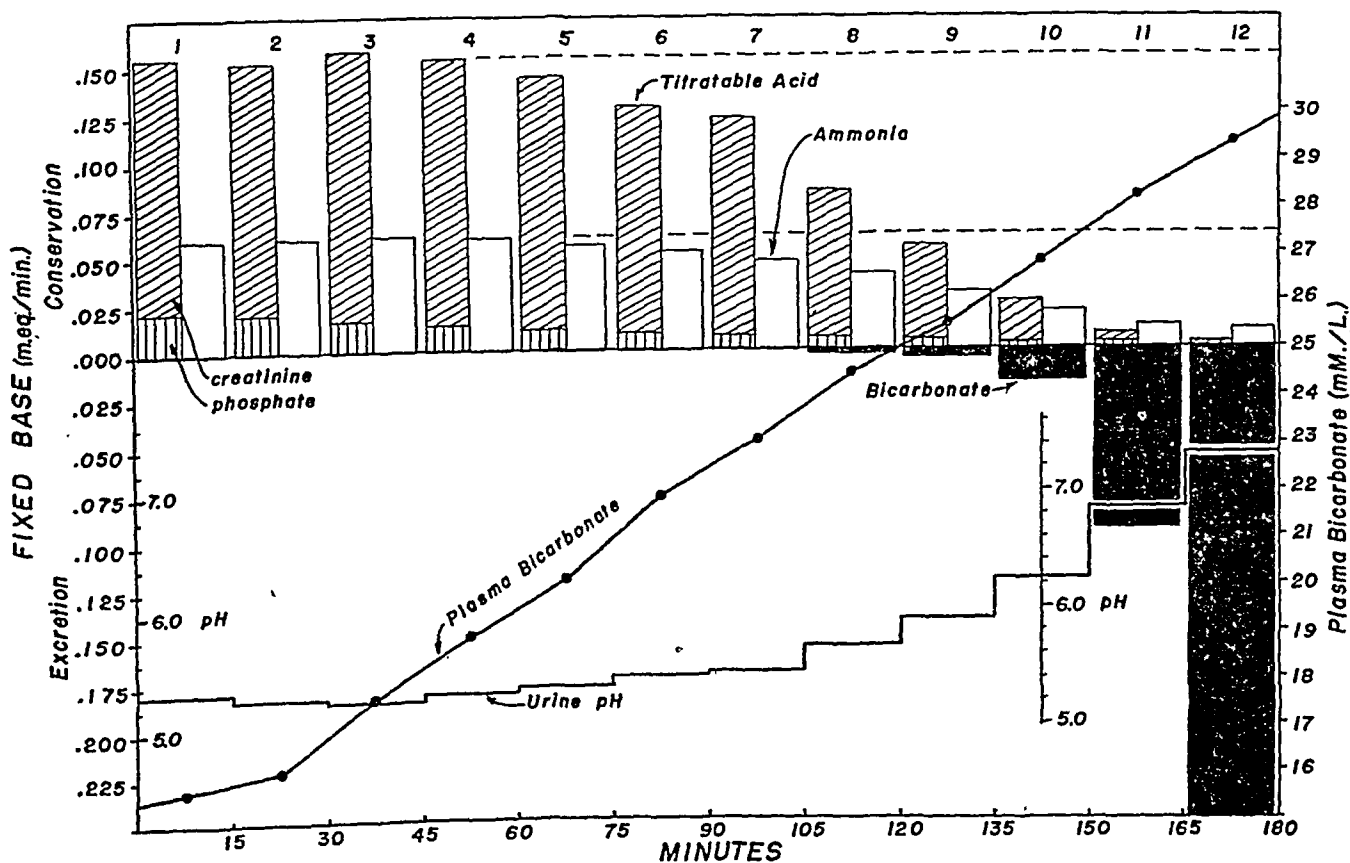


FIG. 4. THE RELATIONSHIP BETWEEN BASE CONSERVATION (AMMONIA AND TITRATABLE ACID EXCRETION), BASE EXCRETION (AS BICARBONATE), AND PLASMA LEVEL OF BICARBONATE

nents to base economy was studied at a series of plasma bicarbonate levels. The results of such an experiment are presented in Figure 4. Since the rate of excretion of buffer substances by the fasted subject in ammonium chloride acidosis is low, creatinine was infused to increase the elimination of titratable acid to levels sufficient for accurate evaluation. Throughout the experiment the rate of excretion of creatinine was maintained at a constant high value. In Figure 4 titratable acid and ammonia elimination are plotted upward, bicarbonate elimination downward, to emphasize their opposite relations to base economy. The titratable acid³ indicated was largely exogenous creatinine, to a slight extent endogenous phosphate as noted on the graph.

During the initial two control periods of this experiment, at plasma bicarbonate levels of 15 to 16 mM per liter, 0.150 mEq of titratable acid and 0.060 mEq of ammonia were excreted per minute. Base economy equalled the sum of these figures, for bicarbonate excretion was negligible. Sodium bicarbonate was then infused and the plasma concentration was raised as a linear function of time over a range of 16 to 30 mM per liter. Acid and ammonia excretion remained at their initial high levels until the fifth period, when at a plasma bicarbonate level of 20 mM per liter, the first perceptible reduction in these components of base economy was evident.

No significant excretion of bicarbonate occurred until the tenth period at a plasma concentration of 26.7 mM per liter, although before this level was reached, titratable acid and ammonia excretion had declined to a fraction of their control values. Thus over the range of 20 to 26 mM per liter the rates of excretion of acid and ammonia decreased in proportion to the increase in the rate of reabsorption of bicarbonate, in the absence of significant excretion of bicarbonate. We interpret this to mean that a type of competitive inhibition of acid and ammonia excretion results from increased reabsorption of bicarbonate. Since acid and ammonia are formed in the distal segment of the renal

tubules (12, 13) and since the final moiety of bicarbonate is reabsorbed from the tubular urine in this same segment (3), we incline to the view that interference occurs at this level. At plasma concentrations less than 20 mM per liter the quantity of bicarbonate delivered into the distal segment is so small that little interference results, and urine of maximal acidity and ammonia content may be formed. However, other factors, especially the duration of the acidosis, affect the excretion of ammonia. Perhaps these factors are the uncontrolled variables which render certain mathematical relationships between base conservation and alkali reserve (14) inexact in some instances.

DISCUSSION

The properties of the renal mechanisms for the reabsorption of bicarbonate in normal man are such as to assure stabilization of plasma concentration at a level of 24 to 28 mM per liter. At plasma concentrations less than 24 mM per liter, essentially all of the bicarbonate delivered into the renal tubules in the glomerular filtrate is conserved, none is lost in the urine. Furthermore acid elimination and ammonia excretion are stimulated as the plasma bicarbonate level falls below 24 mM per liter. Both processes serve to restore base to the body as bicarbonate and thus to rebuild depleted reserves. At plasma concentrations above 28 mM per liter a relatively constant quantity of bicarbonate, namely, 2.8 mM per 100 ml. of glomerular filtrate, is reabsorbed; the excess is excreted in the urine, and plasma concentration returns toward the normal value.

The nature of the tubular mechanisms accomplishing these ends cannot be completely defined at present but sufficient information exists to permit their partial characterization. It is evident that no less than two-thirds (15), and possibly as much as four-fifths or more (16) of the fluid filtered at the glomerulus is reabsorbed during its passage through the proximal segment of the renal tubule. According to Walker (15), the residual tubular fluid and hence the reabsorbate are essentially in osmotic equilibrium with the plasma. Only in the distal tubule does concentration or dilution of the tubular urine occur. Wesson (17) holds that electrolytes are actively reabsorbed in the proximal segment and that the removal of these and

³ Titratable acid values were calculated from the pH of the urine, collected in such fashion as to avoid loss of carbon dioxide, and the measured rates of excretion of creatinine and phosphate. They do not, therefore, include titratable acid contributed by dissolved carbon dioxide.

other osmotically active constituents from the tubular fluid creates a diffusion force which causes the return of water to the blood stream. Thus the composition of the residual tubular fluid varies in its course through the proximal segment owing to the progressive removal of its several valuable constituents and to the progressive concentration of its waste components, yet the osmotic pressure of this fluid remains nearly the same as that of the plasma, for the tubular epithelium is presumed to be freely permeable to water.

In the rat and guinea pig (15) and apparently in the dog as well (17), bicarbonate is reabsorbed relatively more rapidly than is water, chloride or sodium. Thus the concentration of bicarbonate in the proximal tubular fluid falls below, that of sodium remains nearly the same as, and that of chloride rises above its respective level in the glomerular filtrate. If the reabsorption of bicarbonate and chloride were the consequence of passive diffusion conditioned only by electrostatic forces and by the active transfer of sodium, one would expect chloride to be reabsorbed relatively more rapidly than bicarbonate, for the mobility of the hydrated chloride ion is greater than that of the hydrated bicarbonate ion.⁴ The preferential reabsorption of bicarbonate in the proximal tubule therefore suggests its active transport.

This active transport mechanism must be of such nature as to satisfy the following requirements. Ionic electroneutrality of the tubular fluid and reabsorbate must obviously be preserved. The capacity of the mechanism to transfer bicarbonate must be limited in some fashion, for as described above, all filtered bicarbonate in excess of 2.8 mM per 100 ml. is excreted. However, transport capacity, although limited, is not fixed, for at any given plasma level the quantity of bicarbonate reabsorbed is linearly related to glomerular filtration rate (3). These latter two characteristics in reality apply to the overall reabsorption of bicarbonate, but since the major reabsorption of this ion species occurs in the proximal segment, they must be considered to be characteristics of the proximal mechanism. Any further description of the nature of this mechanism is impossible at the present state of our knowledge.

⁴ This statement is based on the specific conductivities of the two ion species derived from the International Critical Tables.

From evidence presented previously (3) as well as that outlined above, the distal tubular mechanism for the reabsorption of bicarbonate would appear to be the same as that which converts urinary buffer salts into free titratable acid. This latter mechanism accomplishes the exchange of hydrogen ions, formed within the tubular cells, for fixed base bound by weak acid anions in the tubular urine. These acid anions are eliminated as titratable acid; the base is returned to the body as bicarbonate (18-20). A similar exchange of hydrogen ions for bicarbonate bound base would form carbonic acid in the tubular urine. Since carbonic anhydrase is absent from the tubular fluid, dehydration of carbonic acid to carbon dioxide would proceed slowly, and, providing any significant quantity of carbonic acid were formed, one would predict that the carbon dioxide tension of the bladder urine would exceed that of plasma.

As shown in the lefthand graph of Figure 3, the carbon dioxide tension of the urine equals or exceeds that of the plasma. As shown in the righthand one the greater the quantity of bicarbonate reabsorbed, the higher the carbon dioxide tension of the urine. We infer that at plasma concentrations less than 20 mM per liter, the bulk of the bicarbonate is reabsorbed in the proximal segment, little is delivered into the distal segment to be absorbed by ion exchange. Accordingly the carbon dioxide tension of the urine is low and approximates that of the plasma. At higher plasma concentrations, proximal reabsorption is incomplete, and appreciable quantities of bicarbonate are delivered into the distal segment to undergo reabsorption by ion exchange. As total absorption exceeds 2.2 mM per 100 ml. of filtrate, the distal contribution becomes appreciable, and the carbon dioxide tension of the urine rises rapidly to a value some three to four times that of the plasma.

The view of the identity of the distal ion exchange and bicarbonate absorption mechanisms is strengthened by the observation, illustrated in Figure 4, that there exists a type of competitive interference between bicarbonate reabsorption and titratable acid excretion, at least at plasma bicarbonate levels between 20 and 26 mM per liter. At plasma levels below 20 mM per liter no interference occurs for the bulk of the bicarbonate is absorbed in the proximal segment and little reaches the level of the distal ion exchange mechanism.

At plasma levels above 20 mM per liter bicarbonate reaches the distal segment in increasing quantities and bicarbonate bound base competes with base bound by fixed buffer acids in the exchange process. Accordingly, the excretion of titratable acid varies inversely with bicarbonate absorption over a range of plasma concentration of 20 and 26 mM per liter. Above 26 to 28 mM per liter frank excretion of bicarbonate occurs and titratable alkali is eliminated in the urine.

A third line of evidence favoring identity of the distal ion exchange and bicarbonate absorption mechanisms is derived from experiments on the dog described previously (3). Sulfanilamide blocks the excretion of titratable acid and reduces the reabsorption of bicarbonate. Presumably it inhibits the enzyme carbonic anhydrase which plays a role in the distal tubular exchange of hydrogen ions for base (18, 19). Bicarbonate reabsorption is only partially blocked by sulfanilamide; presumably it is the distal moiety, entering into the ion exchange mechanism, which is affected.

Although these experiments outline in a general way the relation between plasma concentration, reabsorption and excretion of bicarbonate, they throw no light on the means by which hyperventilation brings about increased bicarbonate excretion. Forced breathing, induced voluntarily or reflexly in consequence of anoxia, lowers the bicarbonate content of the blood, yet leads to the formation of alkaline urine (11). Obviously the capacity of the renal tubules to reabsorb bicarbonate must be reduced, but no evidence exists as to the nature or site of action of the stimulus which brings about this reduction. It is possible that changes in plasma pH may in some way yet to be defined exert a regulatory influence over bicarbonate reabsorption. It is well known that changes in plasma pH of considerable magnitude occur with hyperventilation.

SUMMARY

The renal reabsorption and excretion of bicarbonate have been studied in three normal subjects over a range of plasma concentration of 13 to 38 mM per liter. At plasma levels below 24 mM per liter essentially all of the bicarbonate entering the renal tubules in the glomerular filtrate is reabsorbed. Above 28 mM per liter a relatively con-

stant quantity, averaging 2.8 mM per 100 ml. of glomerular filtrate, is reabsorbed; the excess is eliminated in the urine. Under the conditions of our experiments the renal threshold is 25 to 27 mM of bicarbonate per liter of plasma. Evidence is presented that bicarbonate is reabsorbed by two dissimilar mechanisms, a proximal and a distal one. The latter mechanism appears to be the same as that which converts buffer salts of the tubular urine into free titratable acid. The properties of the proximal and distal tubular reabsorptive mechanisms of man are similar to those previously described in the dog.

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THE BIOASSAY OF ADRENAL CORTICOIDS IN THE URINE OF PATIENTS WITH CONGESTIVE HEART FAILURE

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Fletcher and Schroeder (1) and Warren and Stead (2) suggested that changes in endocrine activity might be present in congestive heart failure and might explain some of the symptoms such as edema and sodium retention. Merrill, Morrison, and Brannon (3) found an increased concentration of renin in the renal venous blood. No other reports of endocrine changes in congestive heart failure appear in the literature. Since sodium and water retention are known to occur following administration of adrenal cortical hormones, especially desoxycorticosterone, studies were undertaken to determine if the urine of patients in congestive heart failure contained increased amounts of corticoid substances.

METHODS

Ten male patients with congestive heart failure were selected at random from the Gallinger Municipal Hospital. These represented four cases of hypertensive heart disease, one of rheumatic heart disease, four of arteriosclerotic heart disease, and one of chronic cor pulmonale associated with asthma. Their ages ranged from 28 to 70 years, and all but two were negroes. The study was restricted to males to eliminate any possible interference due to changes in the menstrual cycle. Six medical students, none of whom had any evidence of heart disease, were used as control subjects. Twenty-four hour samples of urine were collected without preservatives. In the case of the heart failure patients these were collected as soon after admission as possible, usually within the first 48 hours. The first stage of extraction was carried through in all instances within 12 hours after the collection was made.

Urinary corticoids were extracted by the method of Venning, Kazmin, and Bell (4); and the extracted material was stored in the refrigerator until used. Before use this material was taken up in 6 cc. of 10 per cent ethanol so that each cubic centimeter of the resulting solution represented a four-hour excretion of corticoids.

This material was assayed on young male rats of the Sprague-Dawley strain, using the survival time of adrenalectomized rats as one method of assay (5-7). The animals ranged in weight from 70 to 120 gm. Immediately after adrenalectomy these rats were placed on a diet of dog biscuit with 1 per cent sodium chloride and

5 per cent dextrose in their drinking water. After two to four days they were considered ready for use. At 9:00 a.m. of the initial day they were divided into cages of from five to ten rats and placed on a sodium-free diet (Table I) with distilled water for drinking. At 1:00 p.m. of this day they were given subcutaneously their first injection of 0.05 cc. of the alcoholic solution of corticoids. This was repeated at 12-hour intervals as long as the animals survived. With each assay at least one cage was given extract of normal urine and one cage was given 10 per cent ethanol, using the same size dose. Each cage of animals was checked every eight hours for deaths and the survival time recorded in hours.

TABLE I
Sodium-free diet (29)

Casein (vitamin free)	450 gm.
Crisco	200 gm.
Starch	1570 gm.
Dextrose	1750 gm.
Salt mixture*	100 gm.
Distilled water	250 cc.
Thiamine hydrochloride	25 mg.
Riboflavine	50 mg.
Calcium pantothenate	50 mg.
Pyridoxine	25 mg.
Choline	500 mg.
Niacinamide	0.5 mg.
Vitamin A†	0.2 cc.
Vitamin K	10 mg.
Vitamin D‡	0.3 cc.
Alpha tocopherol§	0.1 cc.

* Modified Hubbell salt mixture made without sodium salts (30).

† 250,000 units per cc.

‡ 2 mg. per cc. of calciferol.

§ 50 mg. per cc.

Glycogenic activity was assayed by using male rats weighing 140 to 180 gm. (8). These were adrenalectomized and placed on a diet of dog biscuits and a solution of 1 per cent NaCl. Three days following adrenalectomy these rats were taken off food for 24 hours. Starting at 6:00 a.m. on the day following this fast they were each given 0.20 cc. of the pooled urinary extracts (as described in the results) every hour for seven consecutive hours. One to two hours after the last injection they were given 0.2 gm. of sodium evipal intraperitoneally, and approximately 1 gm. of liver was removed and plunged into hot 30 per cent KOH. Using the method of Good, Kramer, and Somogyi (9) the liver glycogen was isolated and hydrolyzed and the resulting glucose determined by the Shaffer-Somogyi method

TABLE II
Effect of urine extracts on rat survival time

Patient	Age	Diagnosis	Clinical data													Experimental data						
			Duration	Edema	Ascites	Cardiomegally	Hepatomegally	Resp. rate	Orthopnea	Pul. edema	Blood pres.	Pulse rate	Urine	ECG	Remarks	Rat survival time experimental urine	Rat survival time control urine	"t"	"p"			
C.	70	Arteriosclerotic heart disease	2 wk.	+++	0	+		4FB	30	++	+++	130/78	88	Alb. ++	1-3 Heart block, LBBB 1st degree A-V block	Controlled in 9 days	169 ± 59.9* (7)	hrs.				
D.	56	Arteriosclerotic heart disease	2 yr.	+++	+	+		4FB	30	++	+++	150/90	100	Neg.		Controlled in 17 days	162 ± 39.3 (9)	95 ± 21.3 (7)	3.05	<0.01		
N.	26	Rheumatic heart disease	3 mo.	++	0	+++		3FB	18	0	++	105/75	108	Neg.		Controlled in 6 days	110 ± 32.5 (5)					
D.	67	Arteriosclerotic heart disease	6 mo.	++	0	+++		2FB	20	0	+	140/90	84	Sug. +++	Auricular fib. 1st degree A-V block Old ant. infarct	Controlled in 5 days	116 ± 75.9 (6)	110 ± 50.2 (4)	4.28	<0.01		
L.	50	Cor pulmonale	5 yr.	+++	0	+		2FB	45	++	+++	120/80	98	Neg.		Controlled in 8 days	148 ± 19.0 (4)		0	>0.9		
D.	63	Hypertensive heart disease	2 mo.	+	0	+++		0	24	0	++	190/100	108	Alb. +		Controlled 2-4 days	116 ± 75.9 (6)		2.97	<0.01		
.	64	Hypertensive heart disease	6 mo.	+++	0	+++		4FB	40	+++	+++	190/115	100	Alb. ++		Died 5 days after admision.	143 ± 43.2 (6)	105 ± 41.2 (6)	0.233	>0.7		
.	69	Hypertensive heart disease	2 mo.	+	0	0		1FB	30	0	++	200/80	90	Neg.		Controlled in 6 days	131 ± 68.4 (5)	108.8 ± 43.8 (24) (Average)	1.60	0.1		
52		Hypertensive heart disease	4 wk.	+++	0	+		0	26	0	++	140/100	110	Neg.		Controlled in 6 days	103 ± 48.7 (6)		0.627	0.5		
67		Arteriosclerotic heart disease Auricular fib.	3 wk.	+++	0	+++		4FB	24	0	+++	130/90	80	Neg.	Auricular fib.	Digitalis intoxic. Controlled 13 days	131 ± 30.2 (6)	136 ± 39.0 (6)	1.40	0.2		
																167 ± 51.2 (6)		2.38	0.02			

* Standard deviation.
Numbers in parentheses represent number of animals assayed.

* Standard deviation.
Numbers in parentheses represent number of animals assayed.

(10). The results were then expressed as mg. of glucose per 100 gm. of body weight.

The statistical significance of the data was determined by obtaining the value of "P" corresponding to the calculated value of "t" for the difference between the means of the results obtained in the experimental and the control animals (11).

RESULTS

The effect of urine extracts on the survival time of adrenalectomized rats on a sodium-free diet is summarized in Table II. Four urine extracts of patients with congestive heart failure produced a significant prolongation of the survival time of adrenalectomized rats over that of normal subjects when these were assayed simultaneously. The remaining six did not. Correlating this with the clinical findings, it can be seen that these former four patients were sicker than the remainder with one exception, E. J. Three of the four had orthopnea, all had three to four plus peripheral edema, and three to four plus pulmonary edema. Clinically, E. J. would also fall into this group; but his urine did not prolong the lives of the experimental animals significantly. Of the former group, three had arteriosclerotic heart disease and one had chronic cor pulmonale. In the latter group there were four cases of hypertensive heart disease, one of arteriosclerotic heart disease, and one of rheumatic heart disease.

In order to show that adrenalectomized animals would live under the conditions of the experiment if given one of the cortical hormones, 12 adrenalectomized rats were given subcutaneously 0.5 mg. of desoxycorticosterone acetate in propylene glycol twice daily. The procedures used were the same as those used in animals receiving injections of urine extracts. There were no deaths during the desoxycorticosterone injections which were continued for 216 hours. After these injections were stopped the animals lived an average of 194 ± 109.1 hours. The long survival time of these rats subsequent to discontinuing the desoxycorticosterone was possibly due to the slow absorption of the hormone in some animals.

In order to show that the lives of adrenalectomized animals would not be prolonged by the solvent (10 per cent alcohol) alone, 25 animals were given 10 per cent alcohol using the same size dose and procedures as with those animals receiving urine extracts. These animals lived an aver-

age of 108 ± 38.0 hours. This value is approximately the same as the average for the survival time of animals receiving normal urine extracts (95.3 ± 44.3 hours).

In practically all animals receiving the urinary extracts there were observed toxic reactions. Animals became sluggish within two to four days after beginning the injections. Rats weighing less than 70 gm. were not used since it was found that they died very early in a trial run (50.5 hours). Room temperature could not be controlled satisfactorily, and some animals died on cold nights. These factors are probably responsible for the large standard deviations in some instances.

Based on the clinical and experimental findings, the urine extracts were pooled into two groups. Group A consisted of those patients who were the sickest and whose extracts resulted in a significant

TABLE III

Comparison of glycogenic activity of urine extracts from normal subjects and from heart failure patients

Extract	Average liver glycogen (mg./100 gm. of rat)	"P"
Control urine	$0.985 \pm 1.56^* (8)^\dagger$	
Experimental urine:		
Group A	$27.18 \pm 8.27^* (5)$	<0.01
Group B	$24.13 \pm 9.04^* (5)$	<0.01

* Standard deviation of the group.

† Numbers in parentheses represent number of animals assayed.

increase in survival time in adrenalectomized rats (urine extracts of patients C. C., C. D., R. L., and C. Corn.). E. J. was also placed in this group because clinically he belonged there and it was felt the survival time produced by his extracts was similar to that of this group although it was not statistically significant. Group B consisted of those patients who were not so sick and whose extracts resulted in no increase in survival time (urine extracts of patients W. N., I. D., H. D., T. B., and F. S.). These two pooled groups were used in the assay of the glycogenic activity of the urine extracts. The results are given in Table III. Both groups show a marked increase of glycogenic activity over that of control urine extract. The slight difference between the two experimental values was not evaluated because of the small number of animals used.

DISCUSSION

The results of this study indicate that there is an increase in the urinary excretion of corticoids in patients with congestive heart failure. Of the ten patients studied, four who appeared clinically to be the sickest, excreted substances which prolonged the life of adrenalectomized rats while the remaining six did not. There was one patient who clinically belongs in this group whose urine extracts did not prolong the life of adrenalectomized rats. Both of these groups of patients, however, excreted an increased quantity of glycogenic corticoids. An increase of corticoid substances in the urine has been considered as an indication of increased adrenal cortical activity (4-7). There is, however, in these patients another factor which must be considered. All but two of the patients had liver enlargement; and since the liver plays an important role in the inactivation of steroid hormones (12), the question arises that in these cases there might have been enough liver damage to cause only an apparent increase in corticoid secretion rather than a true increase in corticoid production. Some authors (13) report liver dysfunction in a large percentage of patients with heart failure, but this cannot be correlated with the degree of failure (14).

Increased adrenal cortical activity in other instances is believed to be the result of a reaction to various "non-specific stimuli" (15). Of these anoxia has been shown to produce increased adrenal cortical activity (15-18) and Langley and Clarke (16) believe that disturbances in acid-base balance resulting from hyperventilation can stimulate the adrenal cortex. Both anoxia and hyperventilation are present in patients with heart failure and the increase in corticoids found in this study possibly represents adaptation to these non-specific stimuli.

With an increase in adrenal cortical activity sodium retention would be expected, since some of the cortical hormones, especially desoxycorticosterone, act on the renal tubules to promote the reabsorption of sodium from the glomerular filtrate (19, 20).

Other investigations (21-23) indicate that the primary cause of sodium retention in heart failure is a decrease in renal blood flow and glomerular filtration which results from a shunting of blood

away from the glomeruli secondary to renal vascular changes. Merrill (21) and Mokotoff, Ross, and Leiter (22) have concluded that there is no increase in the reabsorption of sodium in heart failure but that its reabsorption is proportional to the glomerular filtration rate. This suggests either that the adrenal corticoid production is not increased sufficiently to affect sodium reabsorption in heart failure or that the adrenal corticoids act on the renal vascular system. Experimental evidence to support the latter is inconclusive. Talbott, Pecora, Melville, and Consolazio (24) studied renal function in patients with Addison's disease and believe that the decreased renal function observed was due to a decreased tone of the efferent arterioles of the kidney and that this was corrected following the administration of desoxycorticosterone. No changes in renal function were observed in normal individuals following the administration of desoxycorticosterone acetate. Waterhouse and Keutmann (25) believe that in Addison's disease desoxycorticosterone produces a vasoconstrictor effect on the vascular system of the kidney. Other investigators have produced changes in the kidney with desoxycorticosterone (15, 20, 26, 27). Swingle (28) believes that desoxycorticosterone may affect the tone of arterioles. At the present time it seems more likely that the increase in corticoid excretion represents a result rather than a cause of the symptoms seen in heart failure. However, because of the close relation between clinical symptoms and the increase in corticoid excretion, further investigation is to be undertaken.

SUMMARY

1. The urine of ten patients in congestive heart failure was studied for the presence of adrenal corticoids and compared with normal control urine.
2. Of these ten patients, four showed an increase of corticoids over that found in normal urine which prolonged the life of adrenalectomized rats.
3. An increase in corticoids with glycogenic activity was observed in urine obtained from heart failure patients over that of urine obtained from normal control subjects.
4. The possible place of increased adrenal cortical activity in heart failure is discussed.

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AUTOANTIBODIES IN HUMAN GLOMERULONEPHRITIS¹

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It has often been suggested that the picture and course of human glomerulonephritis could be best explained by an antigen-antibody reaction. There are many clinical facts which support such a hypothesis. The interval of 10 to 20 days between the primary streptococcal infection and the first appearance of clinical signs of acute glomerulonephritis, the persistent absence of direct evidence of streptococcal invasion of the kidney itself with evidence of severe general involvement of the glomerular apparatus and the often irreversible progress of the disease seem strongly to suggest such a possibility. In our studies on the nephrotic syndrome (1) during the last few years using the fluorescein and other methods for determining capillary permeability, we had the impression that during this stage of chronic glomerulonephritis we are dealing with a diffuse damage of the entire capillary network throughout the body, possibly produced by an "allergic" reaction.

Good evidence has been presented that by an antigen-antibody reaction a picture very similar to human glomerulonephritis and its different stages can be produced in experimental animals. Masugi (2) has shown that a chronic glomerulonephritis can be produced by the injection of a heterologous immune antikidney serum. Sarre and Wirtz (3) were able to demonstrate that such antibodies are rapidly and selectively adsorbed by the kidney and thus disappear out of the circulation, giving rise to specific changes in the kidney. They were also able to show that other organs bind these specific antibodies to a much lesser extent. Swift and Smadel (4) demonstrated the specificity of such antikidney serum by the fact that an injection of kidney extract immediately preceding the injection of the antikidney serum prevented kidney damage. Injection of liver extract did not afford this protection. Burky (5) in turn showed that

lens material of rabbits when incubated with staphylococcus toxin and injected into rabbits produced antibodies to lens which in turn attacked the lens of the same animal species. Hecht, Sulzberger and Weil (6), by the utilization of the synergistic action of staphylococcus toxin and of homologous skin antigen, succeeded in the production of specific antiskin antibodies.

Cavelti (7-9) was able to produce, in a high percentage of rabbits and rats, a picture similar to human glomerulonephritis and rheumatic heart lesions by the injection of homologous kidney and heart extracts, respectively, incubated with streptococci or staphylococci. He also succeeded in showing the appearance of specific antibodies in the blood of such animals with the modified collodion technique of Cannon and Marshall (10). Schwentker and Comploier (11) also demonstrated that in rabbits antibodies to kidney can be produced by injecting a rabbit kidney extract previously incubated with staphylococcus or streptococcus extract. They also showed that in the majority of recent cases of scarlet fever antibodies to kidney can be found by complement fixation one to two weeks after the acute phase but that similar antibodies can be demonstrated only in a small percentage of normal individuals.

Stimulated by these experiments and search for a possible "allergic" mechanism which would explain the increased capillary permeability in acute nephritis and the nephrotic stage we investigated the bloods of 23 patients with different stages of glomerulonephritis and of 68 controls in whom there were no signs of glomerulonephritis by history or clinical examination. Since we deviated in some major points from the collodion method as suggested by Cannon and Marshall (10) and by Cavelti (12) a full description of the method used is essential to enable reproducibility and to avoid if possible pitfalls in the extremely delicate and somewhat unpredictable method.

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METHOD

I. Preparation of collodion particles

All water used in the preparation of collodion particles is triply distilled in glass and all apparatus used is completely made of glass. One-half pound of collodion (non-flexible Merck or Mallinckrodt) is very slowly poured into 2 liters of water with constant stirring. The mass so formed is then washed with four changes of just enough water to cover it. The mass is then dried after excess water has been pressed out with clean hands. The mass is now left to dry for three days in a beaker at room temperature. Thereafter a 5% stock solution is prepared by adding acetone to the mass in a water bath at 40° under constant stirring. This stock solution may be used for three months if kept in a dark bottle.

For a test of 400 tubes 75 cc. of stock solution are needed to obtain the necessary particles. A motor-driven stirring rod is suspended close to the bottom of a beaker containing the stock solution. This is done in a water bath at 40° C. While stirring, a mixture of three parts water to one part acetone is added in a fine stream until a gelatinous mass separates and the supernatant fluid becomes cloudy. This supernate is then discarded and the mass redissolved in a somewhat smaller volume of acetone. This procedure is repeated twice, each time discarding the supernate. The fourth and succeeding supernates are collected in a flask containing 300 cc. cold water. This procedure is repeated until the gelatinous mass fails to form. Compressed air is then bubbled through the suspension until all acetone has evaporated. This requires two days. The suspension is then filtered through glass wool. Subsequently, the suspension is washed by the following procedure: 1) In an angle centrifuge the suspension is spun at 3000 r.p.m. for five minutes. The largest particles settle down and are discarded. 2) The supernate is recentrifuged at 2500 r.p.m. for one hour. The supernate is now discarded and the precipitate resuspended by gentle agitation in 50 to 100 cc. of water to give a milky suspension. 3) Again this suspension is centrifuged at 2500 r.p.m. for one hour. The supernate is discarded and the precipitate resuspended and centrifuged as before. 4) At the end of this third one-hour period the particles are resuspended in the smallest volume of water that will permit complete resuspension and centrifuged at 3000 r.p.m. for five minutes discarding the precipitate. The supernate is used as stock suspension and should not be kept for more than a week. It is recommended that the collodion suspension be examined under the Fisher-Kahn viewer before use for the absence of self-agglutination. If clumps are visible the suspension must be discarded.

II. Preparation of the antigen

The kidneys obtained from autopsies performed within a few hours after death are cut up, washed in several changes of saline until free of blood and ground in a Waring Blendor. Enough 1.1% saline solution made with triple distilled water is added during the grinding

to make a 20% emulsion. Care should be taken that the solution does not overheat during the grinding process. The suspension is then centrifuged at high speed until the supernate is absolutely clear. This takes between two and three hours at 3000 r.p.m. If the solution does not clear at this speed further centrifugation at 5000 r.p.m. should be used. The clear supernate is stored at approximately -20° C. The effectiveness of antigens seems to decrease after 10 days. The antigens differ widely in antigenic properties, a question which requires further study.

III. Coating of collodion particles

After studying many methods of coating the following was found most satisfactory. The final collodion suspension described above is tested for the dilution which is necessary to make it match tube No. 3 of McFarland's scale (13). Small amounts of undiluted collodion suspension and undiluted antigen solution are then mixed in such a way that when the proper amount of 1.1% saline is added to make the collodion suspension match McFarland's tube No. 3 the antigen is in a dilution of 1:60. Various antigen titrations with different antigens and different test sera indicated that an antigen dilution of 1:60 is the most effective dilution although variations with different antigens are considerable. The final mixture of collodion and antigen is permitted to stand at room temperature for one hour before use.

The sera

Bloods to be tested should be collected on the day before use with dry syringes, allowed to clot firmly and the sera taken off. They are kept overnight in an ordinary refrigerator. Prolonged storage of sera shows increasing non-specificity in tests. The last 46 tests were run with the sera inactivated for one hour at 56° C. It is the authors' impression that this procedure eliminates non-specificity but also depresses somewhat the specific titres obtained. It has been accepted at present as part of the routine procedure.

The test

Serial dilutions of the sera by the doubling method and employing 1.1% saline are set up in serological test tubes so that each tube contains 0.5 ml. of the diluted serum. The first tube contains a dilution of 1:5. Then 0.2 ml. collodion-antigen mixture is added to each tube. Controls are made up of each serum (1:5 to 1:40) with 0.2 ml. uncoated collodion in such a dilution as to match McFarland's tube No. 3. Additional controls are made of 0.5 ml. saline and 0.2 ml. of coated collodion particles but no serum. The tubes are stoppered and allowed to stand at room temperature for one hour. Thereafter they are spun in an angle centrifuge for three minutes at 1400 r.p.m. Each rack of tubes is then inverted sharply three times to resuspend the particles and the tubes are then read in a Fisher-Kahn viewer. The positive tubes will show discrete small particles with a clear

surrounding fluid, while the negative tubes show a milky suspension. Numerous modifications of this last method were tested but the method as described above was found to be the most reliable. The use of kidneys of young rabbits as antigens was studied but the tests were too few to be conclusive.

The method is very delicate and cumbersome. Occasionally, entire runs will be negative or positive, without any known deviation in technique. Such runs have to be excluded.

The particular antigen used in our tests seems to be of great importance. It was found that adult human kidneys are much less satisfactory than kidneys of infants or stillbirths. This fact has been demonstrated by us repeatedly and seems rather important not only for the performance of the test but also for the general aspect of the physiologic mechanism. But even kidneys from young individuals vary considerably in antigenic quality for unknown reasons. Fractionation and concentration of the antigen may eventually aid in the explanation of this observation. In order to secure a uniform statistical basis each test was run in such a way that approximately the same number of control and nephritic bloods were used in each run. In 11 tests the sera were absorbed with human liver extracts. The results did not differ from those obtained without absorption. To exclude the possibility of blood group factors influencing the results 11 tests were carried out with absorption of the sera with mixed red cells. Again the results obtained with or without absorption did not deviate, indicating that these factors do not influence the specificity of the results obtained.

RESULTS

Twenty-three cases of glomerulonephritis were examined in 122 individual determinations. The cases were arbitrarily classified as early or late nephritis on the basis of duration of signs or symptoms, a duration of less than one year being considered early and longer than one year being considered late. The 12 cases of early nephritis included four cases of acute glomerulonephritis, four which started with a nephrotic syndrome and four cases of chronic glomerulonephritis of less than

one year's duration. The 11 cases of late nephritis were all chronic glomerulonephritis ranging in duration from one and one-half to 12 years. Six of the late cases had marked reduction of renal function with inulin clearances of less than 18 ml. per minute and chemical retention. In all, 44 determinations were done in the early and 78 in the late cases. The results are given in Table I.

The group of nephritics showed titres of 1:10 or above in 75% of the determinations. The average titre in this group of positive reactions was 1:623. Of the determinations in nephritics, 18% showed a titre of 0, and 7% a titre between 0 and 1:10. The highest titres obtained in this group (1:80,000) were obtained on two occasions from different specimens. Since these values were exceptionally high they were not used as such in the statistics but reduced arbitrarily to the next high value of 1:5000 in order not to raise the average titre disproportionately. Of 23 cases in all stages of nephritis 18, or 78.3% were positive more than 60% of the times tested.

Splitting the group of nephritics up between early and late nephritics the following results were obtained. Of the 12 cases considered as early nephritis with 44 determinations 30, or 68% showed titres of or above 1:10. The average titre was 1:918. A titre of 0 was obtained in 11, or 25% of the determinations; 7% had titres between 0 and 1:10. The 11 cases of late nephritis with 78 determinations showed a titre of 1:10 or above in 61 tests, or 78% of the determinations. The average titre was 1:337. A titre of 0 was present in 11, or 14% of the determinations and a titre below 1:10 but above 0 in seven tests, or 9%.

Of all nephritics examined there was not a single case where on repeated determinations the titre

TABLE I
Antibody determinations in nephritic and control groups

	No. of cases	No. of determinations	Positive determinations		Aver. titre of positive determinations	Cases consistently positive*	
			no.	per cent		no.	per cent
Total nephritis	23	122	91	75	1:623	18	78
Early nephritis	12	44	30	68	1:918	7	58
Late nephritis	11	78	61	78	1:337	11	100
Controls	68	126	24	19	1:78	4	6

* Nephritics 60% or above. Controls 50% or above.

was persistently below 1:10. The age distribution of the nephritics as well as the control cases is given in Table II.

Sera of 68 control cases with various ailments or normals were subjected to 126 determinations (Table I). This group included the following diagnoses: normal young adults, 33; active pulmonary tuberculosis, 11; diabetes, 11; essential hypertension with demonstrable renal involvement, two; essential hypertension without demonstrable renal involvement, three; acute upper respiratory infection, two; Laennec's cirrhosis, one; secondary anemia of unknown etiology, one; syringomyelia, one; inactive rheumatic fever, one; advanced arteriosclerosis, two. The titres in 24

TABLE II
Age distribution of nephritic and control groups

Ages yrs.	Early nephritis no.	Late nephritis no.	Control group no.
0-5	5		6
5-10	1		3
10-20	2	4	21
20-30	2	1	30
30-50	2	6	6
over 50			2

tests, or 19.0% of the control determinations were 1:10 or above. The average titre in this positive group was 1:78. Seventy-six determinations, or 60% of the control tests showed a titre of 0, while 26, or 21.0% showed titres above 0 but below 1:10. Of the 68 control cases four, or 6% were positive more than 50% of the times tested. On each one of these four only one determination was done. It should be stated that of the 68 control cases 26 were tested more than once. In these, 84 determinations were done, of which 20, or 23.8% were positive (1:10 or above). None of these 26 cases were positive in more than 50% of the tests (Table I).

DISCUSSION

From the data described it will appear that in a high percentage of nephritics of all stages of the disease the occurrence of antibodies to normal kidney can be demonstrated. The results described may still be improved in the future since many of the tests were technically imperfect in the beginning and the antigen certainly requires much fur-

ther study with the view of purification. If, in the majority of cases of chronic glomerulonephritis, antibodies to normal kidney tissue are constantly or commonly circulating in the blood the progressive course of the disease would be well explained. Whether the wide variations in titre in an individual case and the occasional complete absence of a positive titre are due to technical imperfections of the delicate method or whether they really represent variations in antibody formation remains to be seen. One case with the nephrotic stage of chronic glomerulonephritis showed a titre of zero during an attack of measles. Subsequently, the nephrotic phase disappeared as is often observed. Two weeks after the measles the titre went up to 1:40. If it is true that as a result of measles the antibody level in general is severely depressed it would possibly explain the temporary clearing of certain phases of chronic glomerulonephritis subsequent to rubeola. The fact that none of the cases of glomerulonephritis showed a consistently negative titre seem to confirm the impression that the mechanism of the disease is related to an antigen-antibody reaction. The presence of high antistreptolysin titres and their increase subsequent to reinfections seem to indicate that the streptolysins may possibly be the factor which split up certain otherwise nonantigenic kidney proteins and combine in a hapten linkage so that they become antigenic. The antibodies in turn are then no longer restricted to the hapten-kidney linkage but attack kidney substance directly in the form of a direct precipitin reaction, as demonstrated *in vitro* by the precipitin reaction with the kidney antigen made visible by the collodion agglutination technique. The high specificity obtained by using infants' kidneys and the marked nonspecificity obtained from adult material may explain the high incidence of glomerulonephritis in young individuals. Further studies to correlate antistreptolysin titres with autoantibodies to kidney are in progress. Rantz and his collaborators (14) have shown that during the first two years of life antistreptolysin titres are very low. This may explain the relatively low incidence of glomerulonephritis in infancy in spite of the high antigenicity of infant kidney tissue. Cavelti's original report that a high percentage of cases of rheumatic fever show antibodies to heart in their sera has recently been modified (15) since tests with a modified antigen

did not show the same degree of specificity. Here again the decisive role of the antigen has been demonstrated, and it is possible that infant's heart may yield better and more consistent results. The low incidence of positive titres in normal controls makes the results more significant.

At present the technical difficulties of the method described in this paper and its occasional complete failure do not make it applicable as a routine clinical test. It is to be hoped that with further knowledge of the antigen new and simpler methods can be worked out for more general use. Our attempts with the complement-fixation method as suggested by Schwentker have failed. The methods as suggested by Hecht, Sulzberger and Weil (6) should be investigated.

One cannot avoid speculating on the possibility of neutralizing these antikidney antibodies as a therapeutic approach if they should prove to be a link in the pathogenesis of glomerulonephritis.

The fact that all authors were forced to employ many injections of their "nephrotoxins" to produce persisting lesions suggests that human nephritis does not develop and progress from a single insult but rather from the constant presence and occasional stimulation of antibodies to renal tissue. This lends support to the thought that a maintained neutralization of the antibodies may arrest or interfere with the progress of the disease.

SUMMARY

1. The clinical and experimental evidence supporting the concept that glomerulonephritis is due to a continuous organ-specific antigen-antibody reaction is reviewed.

2. A modified collodion particle technique for detecting antibodies to kidney tissue is described.

3. In 23 cases of glomerulonephritis of all stages antibodies to human renal tissue were demonstrated in 75% of the 122 tests done (average titre 1:623) and 18, or 78.3% of the cases were positive in more than 60% of the tests.

4. In 12 cases considered as early nephritis 68% of the 44 determinations done were positive (average titre 1:918).

5. In 11 cases considered as late nephritis 78% of the 78 determinations done were positive (average titre 1:337).

6. In 68 control subjects antibodies to human renal tissue were demonstrated in 19% of the 126 determinations (average titre 1:78). Four, or 6% of the 68 cases were positive in more than 50% of the tests.

7. Renal antigens obtained from infants or stillbirths show a greater specificity and higher titres than antigens from adult renal tissue.

8. The importance of the continuous presence of antibodies to kidney in a high percentage of cases of nephritis is discussed in relation to the clinical course of the disease and a possible therapeutic approach is suggested.

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THE HYALURONIC ACID OF SYNOVIAL FLUID IN RHEUMATOID ARTHRITIS

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Rheumatoid arthritis is considered a disease which involves the connective tissue and supporting structures of the body with its primary nidus in the interfibrillar ground substance of these structures (1). The ground substances are thought to be composed of mucopolysaccharide-protein complexes. The nature of two of the polysaccharides is known—chondroitin sulfuric acid and hyaluronic acid. The latter has been isolated from skin, the vitreous of the eye and from synovial fluid (2). The synovial space has been regarded as an enlarged tissue space (3) and synovial fluid may be obtained with relative ease in many patients with rheumatoid arthritis. A study of the hyaluronic acid in the synovial fluid of patients with rheumatoid arthritis in contrast to normals seemed worthwhile as the characteristics of the hyaluronic acid thus obtained might parallel changes in other mucopolysaccharides in the smaller connective tissue spaces throughout the body.

Hyaluronic acid in synovial fluid is the component of the fluid which is responsible for its high viscosity (4). A variety of quantitative viscosimetric methods have been applied to the viscous nature of synovial fluid since 1925 (5). These have yielded conflicting data but most seem to agree that normal joint fluid is a very viscous material (6). Bauer, Ropes and Waine (3) demonstrated that there is a wide range of viscosity values obtained from both normal and pathological fluids. We have shown that the viscosity of normal human knee joint fluid varies inversely with the degree of peripheral edema present (7). When joint fluid or purified hyaluronic acid is diluted with 0.85 per cent saline or distilled water an exponential curve is obtained (8, 9), and with fluids of high viscosity small increments of diluent fluid cause marked decreases in viscosity. For these reasons, viscosity alone cannot be taken as an index of the concentration of hyaluronic acid or of the extent of polymerization of the mucopoly-

saccharide. The concentration of hyaluronic acid may be approximated by the mucin clot method of Bauer *et al.* (3), but the clot thus formed contains both protein and hyaluronic acid and changes in protein concentration could cause errors in the determination of hyaluronic acid concentration. Recently a method has been described whereby the hyaluronic acid content of 1 cc. of fluid can be determined with an accuracy sufficient for a biological method (2).

MATERIALS AND METHODS

Joint fluid was drawn at autopsy from the knees of 11 patients without joint disease. In some of these insufficient fluid was obtained for a 5-cc. viscosimeter. These fluids were diluted with 0.85 per cent saline and the viscosity and turbidimetric determinations of hyaluronic acid were made on the same diluted sample. Fluid was obtained from one or both knee joints of 35 patients with rheumatoid arthritis. Viscosity was determined as described previously (7). Hyaluronic acid concentration was determined by the method of Meyer (2).

The exponential curve derived from dilution of synovial fluid or pure sodium hyaluronate becomes a straight line when the logarithm of the viscosity is plotted against dilution (Figure 1). Dilution mirrors the concentration of hyaluronic acid and thus a straight line relationship exists between the log viscosity and concentration. A quotient obtained by dividing the log viscosity by the concentration would be constant for a given fluid regardless of the amount of diluent extracellular fluid introduced. Since the increased viscosity of synovial fluid is due almost completely to the hyaluronic acid present (7, 10) and the viscosity is an index of the polymerization at a given concentration, this quotient gives an approximation of the mean polymerization of the hyaluronic acid present in a sample of fluid. A highly viscous fluid with a low concentration would yield a high quotient and would indicate an highly polymerized hyaluronate. Thus the extent of polymerization would vary directly with the quotient.

RESULTS

In the normal fluids examined, Table I, the quotient was greater than ten in all. Normally

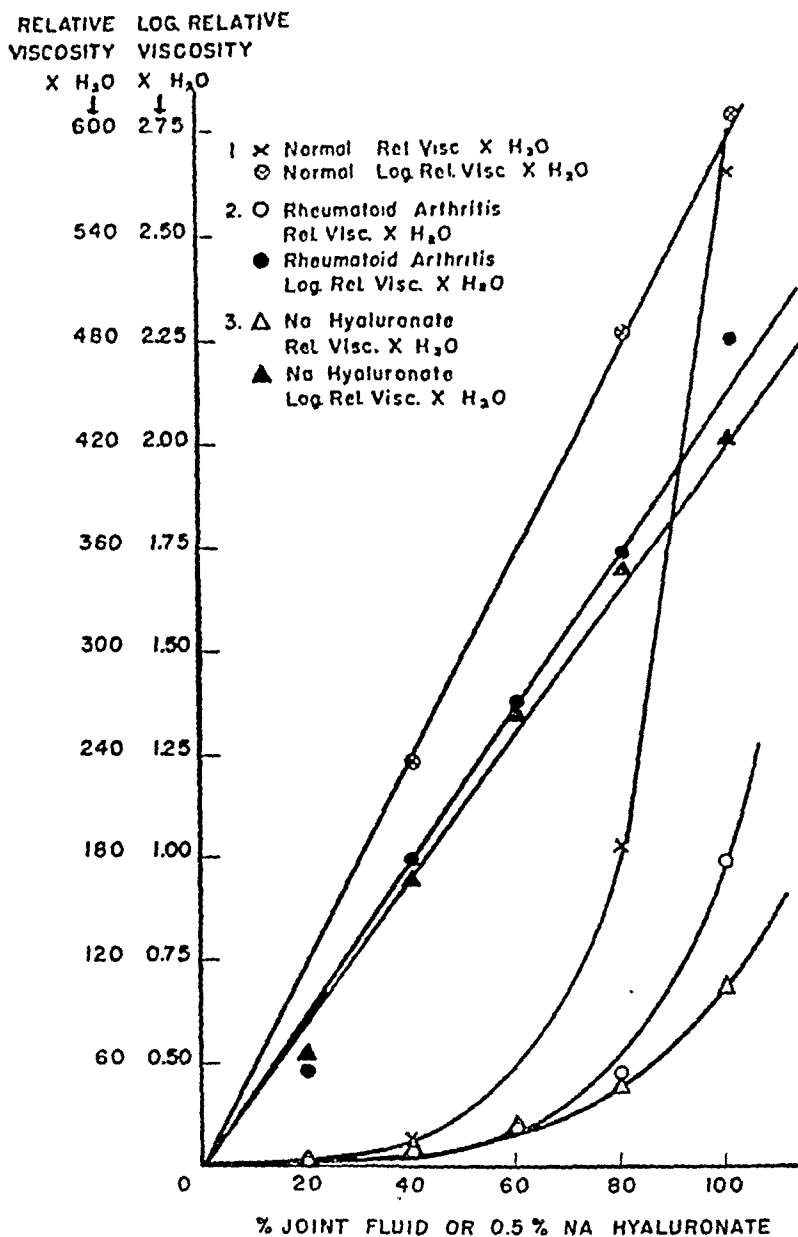


FIG. 1. DILUTION CURVES PLOTTED AGAINST RELATIVE VISCOSITY $\times H_2O$ AND LOG RELATIVE VISCOSITY $\times H_2O$

(1) Knee joint fluid obtained at autopsy from a patient without joint disease. (2) Knee joint fluid from a patient with rheumatoid arthritis. (3) 0.5 per cent Na hyaluronate.

polymerized hyaluronate would have a quotient of ten or above. In the fluid from patients with rheumatoid arthritis (Table II), the quotient was with few exceptions less than ten, which would imply that the hyaluronate in these fluids was less highly polymerized. The severity or intensity of the disease seems to bear some relation to the extent of polymerization of the hyaluronate. With

one exception, patient A. Da., all patients in the group with a quotient above 8.10 exhibited few of the criteria of a generalized illness. With the exception of A. Da., these patients could be grouped as smouldering cases or cases in remission. The four patients with quotients above ten were all what is commonly known as "burned-out cases" of long duration with resultant deformity and few

BLOOD OXYGEN STUDIES IN PATIENTS WITH POLYCYTHEMIA AND IN NORMAL SUBJECTS¹

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During the past few years various studies have been undertaken in this laboratory in an attempt to elucidate the pathological physiology and nature of polycythemia vera. Although the etiology remains obscure, interesting data have been accumulated on (1) blood oxygen saturation, (2) O₂ consumption and CO₂ production, (3) diffusion of gases across the alveolus-blood barrier, and (4) bone marrow findings. The present paper is concerned with a brief discussion of blood oxygen saturation in polycythemia and the results of determinations made on 74 subjects, 48 of whom were suffering from polycythemia vera. Subsequent reports will cover the remaining phases of the investigations and others.

METHODS

The diagnosis of polycythemia vera was made by the usual clinical and hematologic methods which included a complete blood count, packed cell volume, and sternal marrow aspiration. Blood volumes were done on some patients employing the dye T-1824, radioactive colloids of zirconium and yttrium (1), the CO method, or by the use of radioactively labeled red cells. Viscosity determinations and circulation times were also done in most cases. All patients had chest X-rays to exclude pulmonary pathology, and no patient with a chronic cardiac or pulmonary history was included in this series except as noted in the cases of secondary polycythemia.

Blood was drawn from the resting patient anaerobically from either the radial or brachial artery, and the oxygen content and capacity determined by the micro gasometric technique of Roughton and Scholander (2). Arterial capillary blood from the lobe of the ear drawn anaerobically into a tuberculin syringe by means of a small glass ear cup was found to give the same results, and after sufficient controls had been done the ear method

was used exclusively. In this method the whole ear is warmed for one-half hour by placing over it an anesthesia mask containing a small electric bulb. After warming, a small incision with a No. 11 surgical blade will produce a free flow of arterial capillary blood which can be rapidly withdrawn into the syringe containing a drop of mercury and previously rinsed with either heparin or a heparin-fluoride mixture. After the blood is collected, the ear cup is removed from the short rubber tubing connecting it to the syringe, and the tubing is turned on itself and made airtight with a rubber band. The drop of mercury is then used to mix the blood and anticoagulant by rapidly turning the syringe from side to side. The analysis must be made within a few hours, and the syringe with blood must be kept on ice at all times except when the actual determinations are being made.

RESULTS

For purposes of comparison the cases of polycythemia vera were divided into two groups, those with hematocrits below 50% and those above. A total of 48 cases of polycythemia vera was examined on whom 97 oxygen saturation determinations were done, 70 by ear capillary method and 27 by the usual radial or brachial artery method (Tables I and II). Analyses of the oxygen saturation were also done in 18 normal persons (Table III), in five patients with hypochromic anemia (Table IV) and in three cases of erythrocytosis secondary to chronic pulmonary or cardiac disease (Table V).

Eight cases of polycythemia vera had packed red cell volumes below 50%. These patients had been treated with either phlebotomies or radioactive phosphorus before the oxygen determinations were done. The average hematocrit was 44.5 ± 0.62 and ranged from 41.5 to 46.8. The blood oxygen saturation determined on ear capillary blood ranged from 90.0 to 98.4% with an average of $93.9 \pm 1.1\%$. In four of these cases arterial blood from the brachial artery gave a slightly lower oxygen saturation, the results ranging from 88.2 to 93.8% with an average of $91.3 \pm .3\%$.

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³ From the Donner Laboratory of Medical Physics and the Radiation Laboratory.

TABLE I

Results in polycythemia vera with hematocrits of 50% or over

Case No.	Hgb.	RBC X 10 ⁶ /mm. ³	Hem- atocrit	Blood Volume		Ear				Artery			
						O ₂ Content	O ₂ Capacity	O ₂ Satur- ation	Time	O ₂ Content	O ₂ Capacity	O ₂ Satur- ation	Time
	gms.		%	liters RBC	liters plasma	vol. %	vol. %	%	hrs.	vol. %	vol. %	%	hrs.
1	17.6	7.17	60			21.9	22	95	3				
2	24.3	7.96	75			28.8	31	93	4	27.3	30.4	90	5
3	29.8	8.1	76			36.3	37.8	96.2	2	36.0	37.6	95.8	3
4	17.9	6.4	55.8			22.1	23.0	96	1	22.3	23.0	97	2
5	19.0	6.3	57.5			24.4	24.8	98.5	1				
6	20.7	7.96	70			25.0	26.6	94	2				
7	18.5	6.03	55	3.7	3.0	23.7	24.1	98.4	1	23.9	24.3	98.4	2
8	20.1	7.07	60.6	4.4	2.8	25.6	26.6	96.2	1				
9	25.3	9.84	81	9.0±2.0	2.0±0.4	30.4	32.8	92.5	4	30.5	33.2	92.0	4.5
10	22.5	9.22	79	—	—	25.7	27.6	93.0	2				
11	17.3	9.24	62	4.4	2.7	20.0	22.0	91	3	20.2	22.2	91.0	4
12	18.9	5.32	54	3.8	3.3	22.8	24.1	95	4				
13	18.9	5.96	56	4.2	3.5	23.3	24.1	96.8	2				
14	17.0	6.18	60			20.2	22.2	91.0	6				
15	15.2	6.82	50	3.2	3.2	18.9	20.0	92.4	5				
16	23.4	7.82	63			28.7	29.9	96.1	4				
17	15.8	8.66	70			20.3	20.6	98.5	3				
18	22.5	7.76	69			28.3	29.6	95.5	3				
19	18.5	6.24	52			22.0	24.1	91.4	4				
20	22.9	10.82	70			27.0	29.7	92.5	4				
21	23.1	7.62	66	3.9	2.6	27.6	30.0	92	3	28.1	30.0	93.8	4
22	17.3	5.78	50.5	3.2	3.1	21.7	22.2	97.5	2				
23	18.5	7.56	63	4.5	2.5	21.8	24.0	91.0	3				
24	19.2	7.78	70			23.7	24.8	95.5	2				
25	17.3	7.78	55			21.3	22.1	96.5	2				
26	18	6.9	62.5			22.2	23.0	96.5	2				
27	23.5	9.04	72.3			28.9	31.1	93.0	2				
28	19.1	10.2	75.0			23.0	24.6	93.3	2				
29	23.5	9.2	77			28.7	30.8	93.0	1				
30	18	7.09	63			22.8	23.2	98.0	1				
31	15.9	6.24	51			19.5	20.3	96.0	1				
32	19.3	9.85	73			22.7	24.8	91.5	4				
33	16.5	9.62	60			20.4	21.4	95.4	3				
34	15.2	6.7	52			19.2	20	96	3				
35	19.1	6.5	58	4.3	3.4	23.9	25.1	95	1	22.9	24.4	94.0	1.5
36	19.5	7.5	61	4.6	2.8	23.3	25.3	92	2	20.0	21.2	94.0	2.5
37	19.2	6.3	57	4.2	3.1	23.8	25.0	95	1	22.0	23.0	95.5	2
38	20.5	9.2	70	5.4	2.6	25.6	26.7	96	1	26.0	26.9	96.5	2
39	22.0	7.3	97	4.9	2.3	27.7	28.9	96	1	27.6	28.7	96.0	2
40	19.2	6.5	59	4.2	2.9	24.0	24.9	96.5	1	23.8	25.0	95.0	2
Average			63.2					94.7				94.5	

The remaining 40 cases of erythremia had an average hematocrit of $63.2 \pm 1.8\%$ with a mean oxygen saturation of $94.7 \pm 0.36\%$ as determined on ear capillary blood. In 13 of these cases with an average packed cell volume of $67.3 \pm 3.9\%$, the large artery blood oxygen saturation was $94.5 \pm 1.3\%$.

Normal and hypochromic anemia cases examined in the same manner yielded similar results, viz., $95.1 \pm 0.59\%$ and $96.0 \pm 1.1\%$, respectively.

The three cases of secondary polycythemia yielded low oxygen saturation, the results ranging from 75 to 88% with a mean of $79.0 \pm 4.5\%$.

TABLE II
Polycythemia vera with hematocrits less than 50%

Case No.	Hgb.	RBC $\times 10^5/\text{mm}^3$	Hemato- crit	Blood Volume		Ear				Artery			
						O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time	O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time
	<i>gms.</i>		<i>%</i>	<i>liters</i> RBC	<i>liters</i> plasma	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>
1	15.9	7.34	46.8	2.8	3.2	18.9	21.0	90.0	4	18.5	21.0	88.2	5
2	13.9	5.48	45.5			16.4	18.2	90.0	4	16.4	18.2	90.0	5
3	17.5	5.8	42.9			22.6	23.0	98.4	2				
4	18.0	5.62	46.0	3.1	3.6	22.0	23.5	93.5	2				
5	14.9	5.42	41.5			18.6	19.5	93.8	3	18.7	19.6	93.8	4
6	16	5.56	46.0			19.8	20.6	96.0	1				
7	13.4	4.93	43.0			16.3	17.5	93.0	3	16.4	17.5	93.5	4
8	14.9	6.66	45.0			18.8	19.6	96.3	2				
Average			44.5					93.9				91.3	

DISCUSSION

Few complete studies of arterial oxygen saturation in polycythemia vera are available. There is general agreement, however, among the scattered reports that saturation is within normal limits. In a total of 14 reported determinations in uncomplicated polycythemia vera results ranged from 91.0% to 97.0% (3-7), the average of 93.6% being slightly lower than an average of 95.5% reported

for a series of 15 normal individuals (8). These figures compare favorably with those obtained in our study.

Bauer, Lawrowsky, and Skujin suggest after studying a large series of patients, two of whom had polycythemia vera, that there is a lessened degree of oxygen saturation of the hemoglobin as the amount of hemoglobin increases, so that the amount of oxygen carried by a given amount of

TABLE III
Results in normal subjects

Case No.	Hgb.	RBC $\times 10^5/\text{mm}^3$	Hemato- crit	Ear				Artery			
				O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time	O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time
	<i>gms.</i>		<i>%</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>
1	15.6	5.1	46	20.1	20.5	98.2	1				
2	15.8	5.2	46	20.7	20.8	99.4	3				
3	12.9	4.4	42	16.8	17.0	98.9	1				
4	15.6	5.3	47.8	18.6	20.3	90	2				
5	15.3	4.95	44.0	18.7	19.9	94.7	1	18.9	20.1	94.0	2
6	15.8	5.4	48.0	19.4	20.8	93.3	1				
7	15.5	5.2	46.8	18.6	19.0	98.0	1.5				
8	14.3	4.65	42.4	17.1	17.9	95.8	2				
9	13.4	4.45	43	16.7	17.8	93	2				
10	17.0	5.6	48.4	20.8	22.5	92	3				
11	15.1	4.9	44	19.3	20.2	95	2				
12	15.4	4.7	42.5	20.8	23.1	90	1				
13	15.5	4.5	42	16.9	17.7	95	2				
14	14.3	4.7	42	18.4	18.9	97	2				
15	13.6	4.4	43	16.7	17.7	94	3				
16	16.0	5.2	46	19.7	21.2	93	3				
17	15.8	5.25	47	19.4	21.1	92	3				
18	15.3	5.01	45	20.0	20.3	98.5	1				
Average			45.7			95.1					

TABLE IV
Results of oxygen saturation in anemia

Case No.	Hgb.	RBC $\times 10^6/\text{mm}^3$	Hemato- crit	Ear			
				O ₂ Con- tent	O ₂ Capac- ity	O ₂ Satura- tion	Time
	<i>gms.</i>		<i>%</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>
1	11.3	4.05	33.9	14.2	14.8	95.7	1.5
2	11.7	4.5	39.2	15.0	15.5	97.0	2
3	9.6	3.6	26.3	12.3	12.5	98.9	2
4	12.5	4.45	37	15.3	16.1	95.0	2.5
5	11.0	4.2	35.0	13.6	14.6	93	2.5
Average			34.5			96.0	

hemoglobin would be greater in a normal than in a patient with polycythemia (9). In the series of cases studied by us, patients with hematocrits as high as 81% had normal oxygen saturation. Increased oxygen capacity with normal oxygen saturation was reported by Altschule and his co-workers although no figures were shown (10). Moschowitz has used arterial oxygen saturation as the important diagnostic criterion in differentiating between uncomplicated polycythemia vera and secondary erythrocytosis due to hypertension of the pulmonary circulation or prolonged mountain sickness, saturation being normal in true polycythemia (11). Hitzengerger in reporting normal arterial oxygen saturation in six patients with polycythemia vera also stresses this point (6).

It has been suggested by Bansi and Groscurth that the hemoglobin in polycythemia vera does not give up its oxygen to the tissues as readily as does normal blood (12). Isaacs, however, has shown that in polycythemia vera the hemoglobin is normal in all respects, and the oxygen unsaturation of venous blood is within normal limits (13). He found the venous unsaturation to be about 5 vol.%,

the same as the average found by Harrop in 15 normal cases (8). Altschule and his coworkers reported similar results (10).

Fitz interpreted normal arterial oxygen saturation in erythremia as due to increased pulmonary ventilation (14). Harrop and Heath studied alveolar gas diffusion in seven cases by determining the diffusion constant for carbon monoxide, and while finding the blood oxygen content to be high and the saturation low but within normal limits, reported a decrease in the pulmonary permeability to oxygen (3). They measured blood oxygen saturation in three of the patients immediately after exercise, and found significantly lower figures than they had obtained at rest. The failure to maintain normal saturation during a time of increased metabolic demand for oxygen, they felt, was linked to poor pulmonary permeability.

Oxygen saturation of hemoglobin is a function of the partial pressure of oxygen dissolved in the blood. This pressure of oxygen is dependent on the diffusion of oxygen across the alveolus-blood barrier and is in equilibrium with the alveolar oxygen tension of about 100 mm. Hg. Normal oxygen saturation in polycythemia vera would thus seem to signify a normal pressure of oxygen and would further seem to indicate normal diffusion across the alveolar membrane. This is supported by additional experimental evidence to be reported.

It will be noted that the figures for oxygen saturation we report as normal are lower by a small percent than those of some other recent authors. Comroe, for example, in arterial oxygen tension studies reports an average pressure of oxygen of 93 mm. Hg corresponding to about 98% oxygen saturation (15). Direct spectrophotometric studies of the circulating blood of normal dogs by Drabkin and Schmidt have given saturation values of

TABLE V
Results of oxygen saturation in secondary polycythemia

Case No.	Hgb.	RBC $\times 10^6/\text{mm}^3$	Hemato- crit	Ear				Artery			
				O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time	O ₂ Content	O ₂ Capacity	O ₂ Saturation	Time
	<i>gms.</i>		<i>%</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>
1	17.0	6.58	58.0	19.5	22.1	88.0	3				
2	17.4	7.5	63	17.3	23.0	75.0	1.5	17.6	23.0	75.2	2
3	18.5	8.1	65	18.6	24.5	76.0	3				
Average			62			79.0				75.2	

TABLE VI
Results in 12 cases in which low arterial saturations were found

Case No.	Hgb.	RBC $\times 10^6/\text{mm}^3$	Hemato- crit	Blood Volume		Ear				Artery			
						O ₂ Content	O ₂ Capacity	O ₂ Satur- ation	Time	O ₂ Content	O ₂ Capacity	O ₂ Satur- ation	Time
	<i>gms.</i>		<i>%</i>	<i>liters</i> <i>RBC</i>	<i>liters</i> <i>plasma</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>	<i>vol. %</i>	<i>vol. %</i>	<i>%</i>	<i>hrs.</i>
1	14.2	8.25	48	2.9	3.1	16.8	18.9	88.8	18	16.8	18.9	88.8	18
2	15.95	6.74	55			20.3	21.2	96.1	3				
3	15.4	4.78	44			19.6	20.5	95.5	2				
	15.8	5.4	46			18	21.1	85.4	8				
4	14.35	5.39	46.4			16	18.9	85	6				
	18.5	7.51	55			22.6	24.5	92	4				
5	19.3	7.89	57			23.3	25.7	90.5	5				
	14.9	5.88	47			17.7	19.8	89.8	7				
6	17.1	6.2	47			19.3	22.7	85.4	8	20.0	22.8	88	7
	15.0	4.57	42			17.7	19.9	88.4	10	17.2	19.9	86.5	9
7	14.0	5.2	45			16.0	19.1	84	7				
8	15.0	4.53	44			16.8	19.9	84.4	6	16.7	20.0	83.5	6
9	14.5	7.56	46.1			16.2	19.3	84.5	11	16.2	19.4	83.5	10
10	21.0	7.35	65			25	27.9	89.4	9	24.4	28	87	8
11	18.9	7.9	60			21.7	25.2	86	6				
12	15.95	5.75	56			18.3	21.0	87	7				
Average								86.2				83.6	

98.5%; similar determinations carried out on normal human subjects gave figures as high as 98% to 99.3% (16, 17). They concluded that determinations performed by the usual gasometric techniques are too low by about 2% or more.

Ammundsen has shown that normal blood may contain "inactive hemoglobin" equivalent to as much as 2.5 vol.% CO capacity (18). In analyzing the errors of the gasometric method Roughton, Darling, and Root conclude that traces of COHb, which dissociate slightly during oxygenation to increase the effective oxygen capacity, and the conversion to normal hemoglobin of some of the "inactive hemoglobin" described by Ammundsen together with other minor factors give readings which are too low by about 2% (19).

In 12 cases studied at the onset of this investigation, a total of 16 oxygen saturation determinations from ear blood and six from the radial artery were made and the results were very low with an average of 86.2% (Table VI). Only two of the ear determinations were within the normal limits (95.5% and 96.1% O₂ saturation); the remaining 14 results ranged from 84% to 92%. The arterial oxygen saturation results in

six of these cases done at the same time ranged from 83.5% to 88% with a mean of 83.6%. Subsequent determinations in four of these cases revealed normal oxygen saturation results. It was later demonstrated that these low results were due to the fact that the blood was permitted to stand for hours before the oxygen capacity was determined. Correcting this error in technique increased the values and brought the oxygen saturation determinations closer to normal. In polycythemia vera there is an increase in total circulating red cell mass and thus probably an increase in carboxyhemoglobin and in inactive hemoglobin. Unless the O₂ content and capacity determinations are done shortly after withdrawal of the blood, there may be a release of the formerly unavailable hemoglobin with a consequent increase in the oxygen capacity leading to an erroneously low value for the oxygen saturation.

It seems apparent that in the resting patient with polycythemia vera, there is no great deficiency in the saturation of the circulating hemoglobin with oxygen. The values for the oxygen saturation in all the conditions studied except secondary erythrocytosis probably fall within normal limits.

There is an increase in oxygen content and capacity which is proportional to the increased circulating red cell mass. Where the hematocrit is normal, the oxygen content and capacity are similarly normal and the oxygen saturation is about 95%. There of course remains the possibility that during exercise there is significant unsaturation as suggested by Harrop and Heath (3) and this question is now under investigation in this laboratory.

SUMMARY

(1). Arterial blood oxygen saturation in polycythemia vera is within the limits of normal in the resting subject. (2). Low oxygen saturation results may be due to many factors amongst which are the inherent errors of any gasometric technique as well as the presence of traces of carboxyhemoglobin and inactive hemoglobin. (3). Gasometric oxygen determinations must be done soon after the blood is taken to prevent erroneously low oxygen saturation results.

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THE PHOSPHORUS TURNOVER OF CARCINOMA OF THE HUMAN STOMACH AS MEASURED WITH RADIOACTIVE PHOSPHORUS¹

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INTRODUCTION

The phosphorus turnover of tumors has been widely studied in animals and has been found to be higher than in normal tissues. Several blood dyscrasias have also been investigated extensively (1-3). The high uptake of radiophosphorus by leukemic cells and by red cells in polycythemia vera has been applied to the treatment of these diseases (4, 5). Low-Beer and collaborators have observed an increased uptake of radiophosphorus by human breast carcinomas as measured through skin *in vivo* (6); but they have derived no information about turnover. Since no studies have been made of the turnover of phosphorus in naturally occurring human cancers, we thought it of interest to investigate the phosphorus turnover of carcinoma of the human stomach, one of the most common of human malignancies. Because phosphorus takes part in so many reactions of the body, it also seemed of interest to determine whether any change in phosphorus metabolism was related to any particular group of compounds. Therefore, the turnover of the acid soluble, lipid, and protein phosphorus fractions was determined independently. The possible existence of quantitative differences in the phosphorus content of normal tissue and of tumor tissue was also investigated.

METHODS

These studies were made on patients undergoing subtotal gastrectomy, either for malignancy of the stomach or for non-malignant duodenal or gastric disease. The pathological diagnoses and other pertinent data are recorded in Table I. All patients were under the routine care of the surgical service and received blood transfusions and other intravenous medication as indicated.

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Radioactive phosphorus as $\text{KH}_2\text{P}^*\text{O}_4$ was furnished by the Clinton Laboratory at Oak Ridge, Tennessee, and solutions were prepared and standardized by R. F. Cowing of the New England Deaconess Hospital. The original sample was suitably diluted with distilled water and autoclaved. This neutral solution was administered intravenously approximately 36 hours prior to the scheduled time of operation. The chosen dose of 1 microcurie per pound of body weight was based on calculations by Cowing (7) which showed that this would lead to a total body irradiation of not more than 1.5 r.

Within one hour after removal of the stomach the mucosa was stripped from the stomach wall and prepared for analysis. In the non-cancerous stomachs mucosa was taken from the greater curvature, lesser curvature and pre-pyloric area; duodenal samples were obtained whenever possible. In the tumor-bearing stomach the position of the lesion was usually such that normal samples of all the areas could not be obtained. Consequently, a sample of normal appearing mucosa as far from the lesion as possible was taken. Histological study later showed that there were no malignant cells in the areas taken as non-cancerous. In the ulcerating tumors the samples were taken from the actively growing edge of the lesion.

The following analyses were performed:

Total phosphorus: 300 mg of tissue were dried in an oven at 110° C for two to three hours and then digested with 10 N H_2SO_4 and superoxol.

Acid soluble phosphorus (inorganic phosphate, ester phosphate, adenylic acid, adenosine diphosphate, adenosine triphosphate, creatine phosphate, etc.): 400 mg of tissue were minced with scissors and ground with 10% trichloroacetic acid in a small glass Potter-Elvehjem tissue homogenizer.⁴ The filtrate was digested with 10 N H_2SO_4 and superoxol.

Lipid phosphorus (lecithins, cephalins, sphingomyelins, etc.): 500 mg of tissue were finely minced with scissors and ground in the homogenizer with 1:1 ethyl alcohol-ether mixture. The homogenate was then extracted with two successive portions of alcohol-ether mixture on the steambath for periods of 30 minutes each. The combined extracts were evaporated to dryness and digested with 10 N H_2SO_4 and superoxol.

Protein phosphorus (chiefly nucleoprotein, but includes some phosphoprotein which does not yield nucleic acid upon hydrolysis): This fraction was calculated as the difference between the total phosphorus and the sum of the acid soluble and the lipid fractions.

⁴ Made to specifications by Macalaster Bicknell Company, Cambridge, Mass.

TABLE I
Clinical data on patients

Name of patient	Weight	Age	Sex	Time between operation and injection	Pathological diagnosis	Remarks
C. M.	kg. 44.4	54	M	hrs. 40	Inactive cicatrizing duodenal ulcer	Stomach histologically normal
J. M.	59.0	33	M	42	Active duodenal ulcer	
J. C.	61.4	68	M	44	Active benign gastric ulcer	
M. E.	54.4	68	M	45	Polypoid adenocarcinoma of stomach	Lesion size of plum
S. A.	60.4	60	M	38	Ulcerating carcinoma of stomach	Atrophic gastritis
S. K.	73.2	57	M	41	Ulcerating carcinoma of stomach	
M. W.	43.8	66	F	29	Ulcerating carcinoma of stomach	Sample of tissue taken outside of stomach from direct extension of lesion
K. O.	66	60	F	42	Generalized carcinoma-tosis. Origin in stomach	
E. F.	34.4	78	F	24	Linitis plastica	

After the digestion, each sample was diluted to a suitable volume and aliquots were taken of the same sample for counting in solution with a Geiger-Mueller dipping counter (8) and for determination of the phosphorus content by the method of Fiske and SubbaRow (9). The errors of the chemical determination were about 5%, while the counting errors were as high as 10%. The protein phosphorus values, being calculated as differences, have larger errors than the other determinations. Values given in the tables represent averages of duplicate determinations.

Expression of results: Results have been expressed as Biological Concentration Coefficient (10)

$$BCC = \frac{\text{counts per min found in sample/mM}}{\text{counts per min injected/gm body weight}} \times 100.$$

This value is a coefficient and has no dimensions.

All data have been analyzed statistically; Fisher's "t" test was used throughout as a criterion of significance (11).

RESULTS

Phosphorus content: The possible existence of quantitative differences in the phosphorus content of normal gastric mucosa and of cancers was investigated. Table II shows the phosphorus content of the several areas of non-malignant stomachs. Applying Fisher's "t" test to the data in the table, we find that they are statistically homogeneous, i.e., there is no value that varies from the mean of the series by more than could be accounted for by chance. Therefore, the average of these results

TABLE II
Phosphorus content of various areas of non-cancerous stomachs

Name of patient	Total phosphorus				Acid soluble phosphorus				Lipid phosphorus				Protein phosphorus			
	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum
C. M.	mM/gm 0.0668	mM/gm 0.0680	mM/gm 0.0577	mM/gm —	mM/gm 0.0205	mM/gm 0.0205	mM/gm 0.0212	mM/gm —	mM/gm 0.0221	mM/gm 0.0173	mM/gm 0.0123	mM/gm —	mM/gm 0.0213	mM/gm 0.0297	mM/gm 0.0245	mM/gm —
J. M.	0.0706	0.0461	0.0513	0.0674	0.0205	0.0152	0.0168	0.0158	0.0213	0.0102	0.0121	0.0205	0.0289	0.0206	0.0223	0.0316
J. C.	0.0697	0.0436	—	—	0.0237	0.0150	—	—	0.0155	0.0117	—	—	0.0300	0.0169	—	—
Average	0.0600				0.0187				0.0157				0.0232			
S.D.	0.0106				0.00394				0.00474				0.00613			

TABLE III
Phosphorus content of gastric cancer and of non-cancerous gastric mucosa

Name of patient	Diagnosis	Total phosphorus mM/gm tissue		Acid soluble phosphorus mM/gm tissue		Lipid phosphorus mM/gm tissue		Protein phosphorus mM/gm tissue	
		Cancer	Non-cancer	Cancer	Non-cancer	Cancer	Non-cancer	Cancer	Non-cancer
C. M.	Non-cancer		0.0642		0.0207		0.0172		0.025
J. M.	Non-cancer		0.0590		0.0170		0.0160		0.023
J. C.	Non-cancer		0.0568		0.0194		0.0136		0.024
Mean	Non-cancer		0.0600		0.0190		0.0157		0.024
M. E.	Cancer	0.0823	0.0329	0.0202	0.0120	0.0177	0.0100	0.045	0.011
S. A.	Cancer	0.0771	0.0579	0.0234	0.0222	0.0171	0.0146	0.038	0.020
S. K.	Cancer	0.0374	0.0635	0.0224	0.0229	0.0100	0.0162	0.005	0.025
M. W.	Cancer	0.0510	0.0435	0.0159	0.0172	0.0148	0.0121	0.020	0.014
K. O.	Cancer	0.0558		0.0161		0.0114		0.028	
E. F.	Cancer	0.0539		0.0211		0.0136		0.019	
Mean	Cancer	0.0597	0.0494	0.020	0.019	0.013	0.013	0.025	0.017
Overall mean*		0.0597	0.0545	0.020	0.019	0.013	0.013	0.025	0.021
Diff. between means		+0.00516=9%		+0.001=2%		±0=0%		+0.0045=2%	
Probability		$p=0.5$		$p=0.5$		$p=1$		$p=0.5$	

* Mean of all non-cancerous mucosa, regardless of whether stomach bears a cancer.

may be considered to be the mean phosphorus content of the stomach, irrespective of the area of origin.

Table III shows the mean phosphorus content of all the stomachs studied. When non-cancerous stomachs are compared with the non-cancerous portions of cancer-bearing stomachs, there is again no demonstrable difference in the phosphorus content. Therefore, all the non-cancerous mucosae have been grouped as controls and compared with the cancer tissue. The difference in

the means of the cancer and the control group is insignificant. Thus, the phosphorus content is the same for all the tissues studied, whether normal or malignant. The same results are found when each of the phosphorus fractions is studied individually.

Biological concentration coefficient: When the Biological Concentration Coefficient after the injection of radioactive phosphorus is studied, however, a difference between cancer and non-cancerous mucosa can be demonstrated. The Biological

TABLE IV
Biological concentration coefficients of various areas of non-cancerous stomachs

Name of patient	Total phosphorus				Acid soluble phosphorus				Lipid phosphorus				Protein phosphorus			
	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum	Greater curvature	Lesser curvature	Pylorus	Duodenum
C. M.	2976	2821	3069	—	4216	3596	3968	—	2480	2511	2686	—	3007	1054	2511	—
J. M.	2945	3782	3379	3379	5828	5673	6014	5642	3162	3596	3720	2759	1364	2418	3844	2697
J. C.	4929	5890	—	—	6541	9672	—	—	5642	7285	—	—	3379	1643	—	—
Average	3689				5673				3751				2511			
S.D.	104.78				181.04				164.92				954.8			

TABLE V
Biological concentration coefficients of gastric cancer and of non-cancerous gastric mucosa

Name of patient	Diagnosis	Total phosphorus BCC		Acid soluble phosphorus BCC		Lipid phosphorus BCC		Protein phosphorus BCC	
		Cancer	Non-cancer	Cancer	Non-cancer	Cancer	Non-cancer	Cancer	Non-cancer
C. M.	Non-cancer		2945		3937		2541		2201
J. M.	Non-cancer		3379		5797		3317		2573
J. C.	Non-cancer		5394		8122		6448		2511
Mean	Non-cancer		3906		5952		4092		2418
M. E.	Cancer	4495	3689	5,921	5642	5239	3813	3503	1643
S. A.	Cancer	6851	4588	8,711	7099	5332	3782	6448	2511
S. K.	Cancer	5766	3875	7,161	8711	5425	8432	3224	0
M. W.	Cancer	5983	3999	10,323	8215	4743	3968	3379	0
K. O.	Cancer	7533		11,470		8432		4991	
E. F.	Cancer	4526		6,603		5456		1395	
Mean	Cancer	5828	4030	8,370	7409	5766	3968	4030	1054
Overall mean*		5828	3968	8,370	6727	5766	4030	4030	1798
Diff. between means		1860=47%		1643=24%		1746=43%		2232=124%	
Probability		$p=0.01$		$p=0.05$		$p=0.01$		$p=0.02$	

* Mean of all non-cancerous mucosa, regardless of whether stomach bears a cancer.

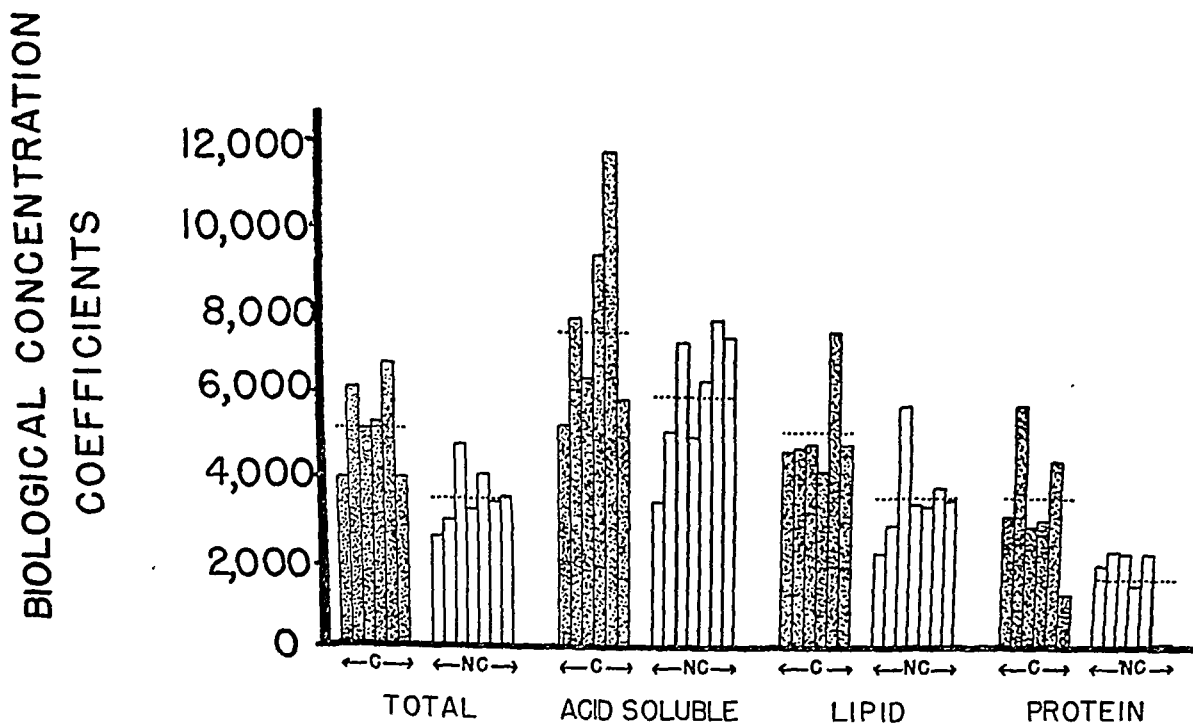


FIG. 1. BIOLOGICAL CONCENTRATION COEFFICIENTS OF PHOSPHOROUS COMPOUNDS IN NON-CANCEROUS GASTRIC MUCOSA AND IN CANCER-BEARING STOMACHS

The dotted lines represent means. C—cancer; NC—non-cancerous gastric mucosa. Each bar represents a patient.

Concentration Coefficients of the various regions of the normal stomach are the same. The use of an average value is therefore justified (Table IV). Furthermore, there is no significant difference between the non-cancerous mucosa from the cancer-bearing stomach and the mucosa from a stomach that does not bear a cancer (Table V). Therefore, all the non-cancerous samples of mucosa are grouped together regardless of presence or absence of cancer in other parts of the stomach, and their average Biological Concentration Coefficient compared to that of the cancer. This comparison (Figure 1) shows an increase in the Biological Concentration Coefficient of the total phosphorus of 47% over the normal ($p < 0.01$). The increase in the Biological Concentration Coefficient of the acid-soluble phosphorus is insignificant, but the 43% increase in the lipid phosphorus and the 124% increase in the protein phosphorus fractions are both statistically significant ($p < 0.01$ and $p = 0.02$, respectively).

DISCUSSION

Since this work was undertaken to study the turnover of phosphorus, *i.e.*, the replacement of phosphorus present by new phosphorus, it was necessary to express results in terms of specific activity—counts per millimole of phosphorus.

Because the radioactivity of a tissue will vary with the administered dose, counts were expressed as fractions of the injected dose. To avoid calculating the decay of the material, the dose was calculated from a standard solution counted on the same day as the samples. It was expressed in terms of body weight in order to compare different patients on the basis of equivalent doses. The factor of 100 is arbitrary. This expression we call Biological Concentration Coefficient.

$$\text{BCC} = \frac{\text{Cpm found in sample/mM P}}{\text{Cpm injected/gm of body weight}} \times 100.$$

The use of this expression is discussed at length elsewhere (10).

The expression Biological Concentration Coefficient has two important properties. It has no dimensions and it is not additive. One cannot add the Biological Concentration Coefficient of the acid-soluble fraction to that of the lipid fraction and that of the protein fraction and arrive at the value for the Biological Concentration Coefficient of the total phosphorus.

Phosphorus content: As pointed out previously, no differences were found in the phosphorus content of normal tissue and of the tumor. This is in contrast to the work of Kishi, Fujiwara, and Nakahara (12) who reported that the lipid phosphorus in experimental hepatomas was markedly decreased. Lustig (13) found a low lipid phosphorus content in human lung tumors and a high inorganic and protein phosphorus content. Whether this discrepancy is due to a species difference or a difference in the type or site of tumors studied is impossible to say.

Turnover: It has been shown that the constituents of a tissue are not static. There is a continuous building up and breaking down of tissue. When a tissue remains of constant size, as many atoms of an element must be lost as are being added. The ratio of the atoms added per unit of time to the total atoms of that element present in the tissue is known as the rate of turnover (14). Although this value cannot be determined absolutely except in special cases, relative rates of turnover can be estimated by comparing Biological Concentration Coefficients.

As labeled atoms are incorporated into a tissue their concentration rises rapidly and linearly at first and then slowly levels off to a maximum value. The maximum will be approximately the same for all tissues (15). During the linear portion of the uptake curve the amount of tracer in the tissue will be proportional to the number of new atoms being added, since the number of labeled atoms present is so small that their loss is negligible compared to the total number lost. Therefore, when radioactive phosphorus is administered to a patient and the Biological Concentration Coefficient is determined soon thereafter, the Biological Concentration Coefficient is proportional to the phosphorus turnover during the experimental period.

In practice we cannot assure ideal conditions. The size of the tissue remains constant within the limits of our experimental error. The ratio of tagged to untagged atoms in the plasma is not constant, but our expression has been so devised that the rate of change is probably the same in all subjects. The ideal time to measure the activity of the tissue (if one could obtain countable samples) would be immediately after injection of the isotope. At this time only a few atoms have been incorporated and the Biological Concentration Co-

efficient will be absolutely proportional to turnover. After this, the uptake curve starts slowly to level off and the Biological Concentration Coefficient will always be less than this value, *i.e.*, it will represent a minimum value. Thus, the differences found are minimum differences, although they approximate the true values.

The rate of phosphorus turnover is at least 45% higher in gastric cancer than in non-cancerous gastric mucosa. This is in accord with the results found in tumors in experimental animals (1-3).

When the acid-soluble phosphorus compounds were studied the turnover rates in cancer and non-cancerous mucosa were found to be essentially the same.

The lipid phosphorus turnover is at least 45% higher in the tumor than in the non-cancerous tissue. Cell phospholipids are present usually as structural cell components so that this increased turnover probably represents a more rapid cell formation and destruction.

The phosphoproteins—chiefly nucleoproteins—are principally cell building blocks. Therefore, the 124% increase in the phosphoprotein turnover as compared to normal turnover probably results from enhanced tissue synthesis. Brues, Tracy, and Cohn (16), studying regenerating liver and hepatoma in the rat, found an increased uptake of phosphorus in nucleic acids and found this to be due chiefly to the desoxyribose nucleic acid, the compound present chiefly in the cell nuclei.

There are two possible ways of explaining the increased growth of a tissue. All tissues are continually breaking down old cells and replacing them with new ones. Normally, in the adult tissue the rates of anabolism and catabolism are equal, so the tissue does not change in size. When a tissue is growing, however, this balance is upset. Either formation of new tissue is enhanced or breakdown of old tissue is inhibited. An increased phosphoprotein Biological Concentration Coefficient represents increased synthesis of phosphoproteins.

Histological evidence of increased mitotic activity would indicate that new cells are being formed. However, new cells might be formed from pre-existing proteins, so that mitotic activity does not necessarily prove that new proteins are being synthesized. Friedberg and collaborators (17) investigated the incorporation of glycine into the tissues of developing embryos and found a high

rate of anabolism. Rittenberg, Sproul, and Shemin (18), on the other hand, studying the incorporation of labeled glycine into proteins of regenerating liver, found that it was incorporated at about the same rate as into the proteins of resting livers. From this they concluded that the mechanism of increasing size was the inhibition of the breakdown of tissue. When studying transplanted rat sarcomas, their results were equivocal (19).

Our results tend to agree with those of the former group since the tumor tissues are synthesizing phosphoproteins at more than twice the normal rate. Catabolism must also be increased to some extent, since the increased rate of turnover is much higher than the observed rate of growth. These experiments then, tend to show that the increasing size of a tumor is due to the increased building of cell constituents and an increased breakdown which, however, is not rapid enough to maintain a constant normal size.

Clinically, it has been recognized that on some portions of the stomach, such as the pre-pyloric area and the greater curvature, cancer is common. On the other hand, cancer is less common on the lesser curvature, and is very rare in the duodenum. No satisfactory explanation has been advanced for this. The possibility suggested itself that in the absence of cancer, the regions in which cancer is common had a metabolism more like cancer than did those regions where cancer is rare. The results of studying the few non-cancerous stomachs available tend to show that the rates of phosphorus turnover in all areas of the stomach are the same. Of course, completely normal stomachs are not available as operative specimens.

Another common clinical observation is that cancer of the stomach and atrophic gastritis occur together more frequently than would be expected from their occurrence alone in the general population. This has led to the suggestion that atrophic gastritis might be a precancerous lesion. If this were true, one might expect that the atrophic mucosa would show chemical or metabolic properties intermediate between the cancer and the normal. Cowdry (20) observed this to be the case with many properties of experimental and naturally occurring cancers of the skin. To investigate this possibility the uninvolved mucosa of cancer-bearing stomachs (usually showing atrophic gastritis) was compared with the mucosa of non-cancer-bearing

ing stomachs. At least in regard to the rate of turnover of phosphorus, no difference could be observed. The evidence tends to show that the malignant type of metabolism is limited to the confines of the tumor itself, and that the rest of the mucosa behaves normally.

SUMMARY

The phosphorus content and the rate of phosphorus turnover of non-cancerous gastric mucosa and of gastric carcinoma have been measured with the aid of radioactive phosphorus.

1. The rate of turnover of phosphorus by gastric carcinoma is at least 45% higher than by non-cancerous gastric mucosa.

2. There was at least a 124% increase in the turnover of protein phosphorus and at least a 45% increase in turnover of lipid phosphorus; the acid-soluble phosphorus turnover remained unchanged.

3. The increased rate of phosphorus turnover is limited to the cancer itself and is not demonstrable in the surrounding tissue.

4. In the non-cancerous stomach there are no differences in those areas in which cancer is common and in those in which it is rare.

5. There is no difference in the phosphorus content of cancer of the stomach and non-cancerous gastric mucosa.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE
PRODUCTS OF HUMAN PLASMA FRACTIONATION.
XXXVII. THE METAL-COMBINING GLOBULIN
OF HUMAN PLASMA¹

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The separation of the proteins of human plasma into a series of fractions and subfractions, uniform with respect to certain physical properties, has generally led also to the separation of proteins with respect to their chemical and biological properties. Among the properties that have long been recognized has been the binding of metals by the plasma so that the metals were no longer freely dialyzable (1). When plasma fractionation was undertaken, early in the war, the Committee on Blood Substitutes of the National Research Council were informed that "investigations of the distribution of . . . metals known to be combined with proteins in the blood, such as copper, zinc, and iron, are planned and will be subsequently reported, as will studies upon the hormones and other physiologically important components of human plasma for which methods of bio-assay are available. We feel keenly that these should now be studied in the large amounts of human plasma fractions becoming available as by-products of the preparation of human albumin for transfusion in shock" (2).

¹ This paper is No. 72 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Mass., on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross. This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August 1941 to July 1946 it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institute of Health.

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Clinical studies had suggested not only that there was a binding of iron and copper, but that there might be a relation between iron and copper binding by the plasma proteins in the sense that an increase in the concentration of the one metal in the plasma was often associated with a decrease in the other (3-5).

The next advance in our knowledge of the binding of metals by proteins followed bacteriological investigations upon egg white. Schade (6) reported that the growth of the bacterium *Shigella dysenteriae*, which requires iron for its metabolism, was readily inhibited by egg white. The same phenomenon of inhibiting bacterial growth by the binding of iron by protein was next noted as a result of studies upon human plasma (7). Fractions of human plasma were made available to Schade, at his request, and the property of bacterial growth inhibition found to be completely concentrated in the fraction that had been designated IV-4 (8).

The methods of detecting this protein component colorimetrically that were being developed by Schade were adopted, with slight modifications, for our studies, and led to the concentration of the iron-binding protein component of plasma into a new fraction (IV-7) (9), and finally to its crystallization (10).

This component of plasma has been designated the β_1 -metal-combining globulin because of *in vitro* evidence, obtained with the crystallized protein, that it is capable of binding copper and zinc, as well as iron. The clinical and physiological studies reported in this series of communications have been carried out with Fraction IV-7, which is readily prepared in large amounts as a by-product of the fractionation of human plasma to yield serum albumin, γ -globulin, fibrinogen, thrombin and other stable blood derivatives, rather than with the more highly purified crystallized protein.

Fraction IV-7 consists to the extent of 76 per cent of the metal-combining protein; the inert components being mainly albumin and an α_2 -globulin.

MATERIALS AND METHODS

The Fraction IV-7 and crystallized β_1 -metal-combining globulin used in these studies were prepared from Fraction IV-4 from blood collected by the American Red Cross by methods to be described elsewhere (8, 9).⁴

Carbohydrate analyses were performed by a modification of the method of Sørensen and Haugaard (11), using orcinol. An equimolar solution of mannose and galactose was used as the standard.⁵ Electrophoretic analyses were made in 0.1 ionic strength sodium diethyl barbiturate buffer at pH 8.6.⁶

Assays for metal-combining activity were made with a modification of the method of Schade and Caroline (7) by spectrophotometrically determining the colored iron-protein complex. To aliquots of a solution of the protein in pH 7.4, ionic strength 0.1 barbiturate buffer were added different amounts of a standard solution of ferrous ammonium sulfate. After standing 60 minutes at room temperature the optical densities of the solutions were read in the Beckman spectrophotometer at a wavelength of 465 m μ . On plotting optical density as a function of iron added, it was found that the extinction increased linearly to a maximum after which it remained substantially constant. The intersection of the two straight lines plotted through the points was taken as the most accurate estimate of the combining capacity. We are greatly indebted to Dr. Schade for his continuing collaboration as well as for periodic microbiological assays which confirmed the results of the spectrophotometric determinations.

RESULTS

A. Studies with the crystallized β_1 -metal-combining globulin

The isolation of a pure crystallized protein with all the chemical and physiological properties of the metal-combining globulin proved the identity of the protein. We have therefore based our stud-

ies on the properties of the crystallized globulin. The behavior of less pure concentrates of the globulin, such as Fraction IV-7, should differ only quantitatively with respect to properties attributable to the specific component interacting with iron or other metals. Those properties observed with a concentrate, not attributable to the pure globulin, should therefore be attributed to impurities in the concentrate removed during the further purifications and crystallization.

1. Physical and chemical properties

Electrophoretically, the metal-combining globulin is characterized as a β_1 -globulin. The protein of plasma has been estimated to contain 11 per cent of β_1 -globulins (12) of which approximately 3 per cent is the metal-combining protein. The latter differs in size, shape, solubility, and composition, from the other β_1 -globulins of the plasma which are largely separated into Fraction II + III (13). The metal-combining globulin resembles the albumins more than most of the other globulins in its solubility behavior and its stability under a variety of conditions of pH, ethanol concentration and temperature. Unlike the albumins, it is in an isoelectric condition near pH 5.9. The sedimentation constant, $S_{20,w} = 5.0$, and molecular weight of 90,000 indicates a molecule of somewhat larger dimensions than the albumins (10, 14). The metal-combining globulin contains but a negligible amount, if any, of lipid. Its carbohydrate content has been estimated to be 1.8 per cent. The nitrogen content was 14.7 per cent, corresponding to a nitrogen factor (gm. protein/gm. nitrogen) of 6.82. The latter is somewhat higher than that of the albumins and many other proteins, and undoubtedly reflects the presence of carbohydrate in the molecule.

2. Interactions with iron

Solutions of the complex formed between the crystallized globulin and iron exhibited the salmon red color which Schade (6, 7) had observed to be characteristic of fractions of plasma active with respect to the microbiological test, and indeed, of the iron-binding protein of egg white. The visible absorption spectrum of the iron-protein complex, measured against a protein blank, is represented

⁴ Certain of the preparations of Fraction IV-4 were derived from out-dated dried plasma, being fractionated for the American Red Cross by E. R. Squibb and Sons, New Brunswick, New Jersey, and were released by the American Red Cross for this investigation. For other preparations of Fraction IV-4, fractionated from fresh plasma by the Division of Biologic Laboratories, Massachusetts Department of Public Health, we are indebted to Drs. V. A. Getting, G. Edsall, and D. J. Mulford.

⁵ We are grateful to Dr. D. M. Gibson and Mr. Robert S. Gordon, Jr., for the carbohydrate analyses.

⁶ We are indebted to Mr. M. J. E. Budka and Miss M. M. Hasson for these measurements.

in Figure 1.⁷ The broad absorption band had its maximum at 465 $m\mu$, with $E_{1\text{ cm.}}^{1\%} = 0.570$.⁸ This band was the basis of the spectrophotometric determination of iron-binding capacity, described previously. The width of the band allowed use of a number of wavelengths other than the maximum for the determination. Thus, Rath and Finch (15) have used the absorption at 525 $m\mu$ in their titrations of serum to avoid interference by other substances present which absorbed at shorter wavelengths.

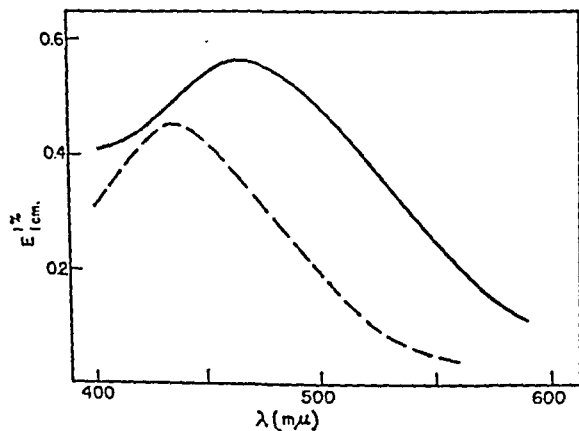


FIG. 1. ABSORPTION SPECTRA OF METAL-COMBINING GLOBULIN IN PRESENCE OF IRON AND COPPER, MEASURED AGAINST A PROTEIN BLANK: *Solid line*—COMPLEX WITH IRON, pH 7.4, $I/2$ 0.1 BARBITURATE; *dashed line*—COMPLEX WITH COPPER, pH 8.5, $I/2$ 0.05 BARBITURATE

Neither the iron-free globulin nor iron alone absorbed appreciable amounts of light at these wavelengths and in the concentration range used. Indeed, the formation of a colored complex with iron was a unique property of the metal-combining globulin, to the exclusion of all the other plasma proteins. This was confirmed by observation of the migration of a solution of the complex in the Tiselius apparatus at pH 8.6. The color was found to move in sharp coincidence with the peak

⁷ Preliminary studies suggest that in the ultraviolet region, iron produces a non-specific enhancement of the usual protein absorption near 280 $m\mu$. This has been observed also with bovine serum albumin.

⁸ The extinction coefficient, $E_{1\text{ cm.}}^{1\%}$, is a constant at any given wavelength, and is defined by the expression: $\log I_0/I = E_{1\text{ cm.}}^{1\%} \cdot c \cdot l$, where $\log I_0/I$ is the optical density, c , the concentration in gm./100 ml., and l , the length of the path of light through the solution in the cuvette, measured in cm. This is the familiar Beer-Lambert equation.

of the protein Schlieren diagram throughout the duration of the experiment.

The reaction between iron and the metal-combining protein was dependent on pH. At neutral pH, above pH 6.5, the color and interaction were maximal. On acidification the color diminished, and below pH 5 the iron could be dialyzed from the solution. The dissociation of the complex and the loss of color were readily reversed by readjusting the system to pH 7. This dependence of the metal-protein interaction on pH is represented in Fig-

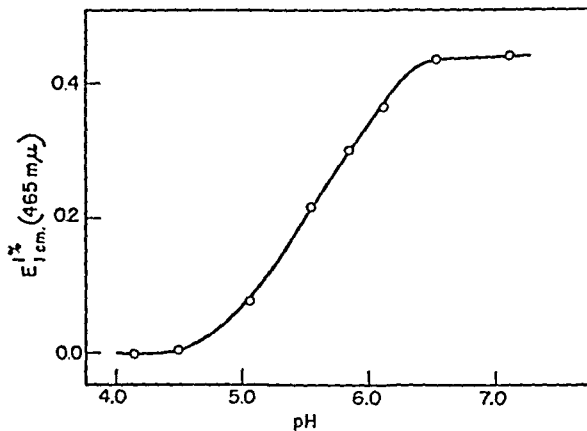


FIG. 2. SPECTROPHOTOMETRIC BEHAVIOR OF METAL-COMBINING GLOBULIN IN PRESENCE OF EXCESS IRON AS A FUNCTION OF pH

Iron (as $\text{FeSO}_4 \cdot [\text{NH}_4]_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$) 2.8 γ/mg . Fraction IV-7. Measured against protein blank.

ure 2. The experimental points were obtained by titrating aliquots of an acidified solution of Fraction IV-7, containing ferrous ammonium sulfate, with alkali in the absence of buffer. The amount of iron in each aliquot was the same and corresponded to approximately two-fold excess over that which could be bound to the protein at the pH of maximum binding. The optical density and pH of each aliquot were measured after standing overnight at 5° C.⁹

⁹ We have not attempted to evaluate the mass law equilibrium constant for the interaction because of the number of variables involved. However, at neutral pH, under conditions of maximum combining capacity, the interaction of the protein with iron was practically independent of the concentration of free iron in solution. Were this not so, the titration curve of the protein with iron would not have shown the sharp "break" at the point of saturation. At acid pH, this was not so, and the titration curves were of sigmoid shape.

The crystallized globulin was capable of binding 1.25 γ of iron per milligram of protein. This corresponded to two atoms of iron per molecule of protein of molecular weight 90,000. Although we have preferred to use ferrous salts for studying the interaction, iron in the form of ferric salts also interacted with the protein. The complex so formed was identical in its spectral absorption to that formed by the use of ferrous salts. The interaction with ferric salts was much slower, however, possibly because of their lower solubility and more rapid hydrolysis at neutral pH. Further evidence of the identity of the complexes formed with ferrous and ferric iron was obtained by Dr. Michaelis (16), who measured the magnetic susceptibility of the complex formed between the crystallized metal-binding globulin and ferrous ammonium sulfate. The results indicated that the iron in the complex is in the ferric state and that the complex is ionic, not covalent. It is therefore evident that the ferrous iron was oxidized prior to or during the formation of the complex with the protein.¹⁰

The iron-binding capacity of Fraction IV-4, or any of its subfractions which contained metal-binding globulin, has always been found equal, within the limit of error achieved, to the expected iron-binding capacity of the crystallized material on the assumption that all of the electrophoretically determined β_1 -globulin in the fraction was the metal-combining globulin. Since no other fraction of plasma contained measurable quantities of the globulin, *we estimate that normal plasma contains 2.4 gm. per liter of this component, capable of binding a total of approximately 3 mg. of iron per liter.* This is in good agreement with the value of 315 γ per 100 ml. found by Rath and Finch (15).

3. Interaction with copper

The crystallized metal-combining globulin also interacted with copper in a manner similar, in most

respects, to the reaction of the globulin with iron. The copper-protein complex had a green color whose absorption spectrum has been represented in Figure 1. The maximum was near 435 $m\mu$ with $E_{1\%}^{1\text{cm.}} = 0.45$. Copper-binding capacity was determined by titration with a standard solution of copper sulfate. A pH 8.4 barbiturate buffer of ionic strength 0.05 was used. Under these conditions each milligram of protein bound 1.4 γ of copper, corresponding to two atoms of copper per molecule of protein.

The interaction of copper with the crystallized globulin differed from that of iron in that the pH-binding curve for the copper was displaced approximately 1 pH unit toward alkaline reactions relative to that of iron. Maximum binding occurred near pH 8.5; at pH 7, where the protein had its maximum binding capacity for iron, its capacity to bind copper was only approximately half maximum.

It was of interest to study the relative affinity of the metal-combining globulin for copper and iron. Using the absorption spectra as an indication, no conditions have been observed under which copper displaced iron from the iron-protein complex. Further, below pH 8, iron displaced copper from the copper-protein complex. Copper and iron are therefore probably bound to the same groups on the protein molecule, the affinity for iron being higher at neutral pH.

Because of these results of *in vitro* studies we have designated this component of plasma the metal-combining globulin.¹¹ While the evidence from clinical studies (15, 18) has suggested that this protein is indeed responsible for the physiological transport of the iron in plasma, and the chemical studies have shown that this component was present in sufficient concentration to satisfy the iron-binding capacity of plasma, the evidence for the *in vivo* transport of copper is less clear. Thus, while chemical studies have shown that this component was capable of binding copper to a certain extent at physiological pH, they do not preclude the existence of a separate *in vivo* mechanism for the transport of copper.¹² Indeed, the binding of copper by albumins has been shown by Klotz (19).

¹⁰ Studies on the time necessary for full development of color suggested that autooxidation of ferrous iron was a prerequisite to binding (10). Thus, at pH 8, full color developed within a few minutes, whereas at pH 6.5, several hours were necessary. Schade, Reinhart and Levy (17) have found that carbon dioxide has a role in the formation of the complex. Other factors may be involved as well (22).

¹¹ This component has been called "siderophyllin" by Schade, Reinhart and Levy (17) and "transferrin" by Holmberg and Laurell (23).

¹² Since this manuscript was submitted, the work of Holmberg and Laurell (23) has come to our attention.

4. Interaction with other metals

Of a series of metals investigated, including cobalt, nickel, zinc, manganese, as well as copper and iron, only the latter two formed a complex with the protein, as characterized by an absorption of light. However, failure of formation of color cannot be considered as evidence of failure of complex formation. Thus zinc, which failed to produce a color when added to solutions of the metal-combining globulin, was nevertheless bound to the protein at neutral pH as shown by direct analysis for zinc.¹⁸ Studies on the interactions of the globulin with zinc and other metals will be the subject of later communications.

B. Properties of Fraction IV-7 of human plasma

In the subfractionation of Fraction IV-4, the metal-combining globulin was further purified and concentrated in Fraction IV-7, the fraction recommended for use in clinical studies on this component of plasma. The preparations which were fractionated at the Harvard Pilot Plant contained an average of 76 per cent of the metal-combining globulin. They differed with respect to this component only quantitatively from the crystallized material. Thus, Fraction IV-7 bound 0.8 to 0.9 γ of iron per milligram of protein.

The impurities in Fraction IV-7 were mainly albumin and α_2 -globulin. Certain properties of this fraction were due to these impurities and were absent in the crystallized metal-combining globulin. Among these were an ability to bind bilirubin (20) and hematin (21). The binding of bilirubin was higher than could be attributed to the albumin present, and was found to be a characteristic of the α_2 -globulin in the fraction. This latter component of Fraction IV-7 was thus responsible for the occasional presence of colored impurities in preparations of Fraction IV-7 derived from hemolyzed plasma. Highly colored samples from this source have, however, had unimpaired metal-binding capacities.

They confirm many of our findings and suggest further that the main part of the serum copper is normally associated with an α -globulin which is insoluble in 50% ammonium sulfate.

¹⁸ We are grateful to Dr. B. Vallee for the zinc analyses.

Solutions of Fraction IV-7 for clinical use have been made by dissolving the dried protein in isotonic saline at pH 6.8 in a concentration of 25 gm. of protein per 100 ml. of solution. Neither acetyl tryptophane, which has been used to stabilize solutions of albumin, nor glycine, which stabilizes γ -globulin solutions, were as effective as sodium chloride in stabilizing solutions of Fraction IV-7. Such solutions were capable of binding approximately 22.5 mg. of iron per 100 ml. and were therefore concentrated approximately seventy-fold over plasma with respect to the metal-combining globulin.

SUMMARY

1. A component of normal human plasma, the metal-combining globulin was concentrated in Fraction IV-7 and crystallized therefrom.
2. The metal-combining globulin is a β_1 -globulin and is distinct in its behavior and properties from other β_1 -globulins of plasma.
3. The capacity of this component of plasma to bind metal ions, particularly iron, copper and zinc, has been discussed. These interactions with metals were readily reversed by change in pH and, in the case of copper and iron, were characterized by the formation of colored metal-protein complexes with characteristic absorption spectra.
4. The protein had a higher affinity for iron than for copper in solution as judged by the capacity of the former to displace copper from the complex.
5. Fraction IV-7 derived from human plasma has been recommended as a concentrated source of the metal-combining globulin. Its safety for injection in man has been investigated and its properties reported.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXVIII. SERUM IRON TRANSPORT. MEASUREMENT OF IRON-BINDING CAPACITY OF SERUM IN MAN¹

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Iron absorbed from the intestine, from destroyed erythrocytes, and from storage depots must be constantly redistributed via the blood stream to satisfy the needs of various body tissues. The studies of Heilmeyer and Plotner (1) and of Moore and his associates (2) indicate that the iron of the serum performs this function. It has been further established that this iron is protein-bound, since the iron is non-dialyzable (3), does not appear in the ultrafiltrate unless acidified (4) and is precipitable with the serum globulins (5).

Employing a micro-biological assay method, Schade (6) recently localized an iron-binding protein in Fraction IV-4 of Cohn and his associates. The crystallized protein (7) is a β_1 globulin⁴ with a molecular weight of approximately 90,000, and binds two molecules of iron per molecule of protein. The globulin itself is colorless but when combined with iron, develops a salmon red color. This color reaction described by Schade has been utilized in the measurement of the iron-binding capacity of normal and pathological sera.

METHOD

Schade (6) has shown that a progressive development of red color occurs on the addition of iron to this β_1 globulin until the protein becomes saturated. At the point of saturation there is a sharp break in the color

¹ Supported by a grant-in-aid from the United States Public Health Service.

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⁴ This β_1 globulin has been variously termed metal-combining globulin and iron-binding protein; the terms being used synonymously. *In vitro* evidence indicates that the protein is capable of combining with other metals (8). Since the only *in vivo* function of this protein thus far demonstrated is that of iron transport, reference to other metal binding will not be made in this paper.

curve which corresponds to the exact point at which free iron may be demonstrated by bio-assay. The spectrophotometric absorption curve of this iron-protein combination has been described (8). A wave length of 525 $m\mu$ was arbitrarily chosen in our studies because of the greater color absorption of serum at shorter wave lengths. On each sample of serum a determination of serum iron and unsaturated iron-binding capacity was made.

Fasting venous blood is drawn without hemolysis into a syringe coated with mineral oil. The clotted blood is centrifuged at 2,000 r.p.m. for 15 minutes and the serum obtained is recentrifuged to remove all red cells. The Coleman Spectrophotometer, Model 11, and cuvettes of 1 cm. depth are used. One cuvette is filled with 5 cc. of 0.9 per cent saline, while in the other is placed 2 cc. of serum and 3 cc. of 0.9 per cent sodium chloride. Originally each cuvette was filled with serum, one serving as a blank. This was found to be unnecessary. Iron standard solution⁵ was added in 0.05-cc. quantities to both cuvettes and a glass stirring rod used to mix the contents of the cuvette after each addition of iron. Readings of per cent of light transmission are made two or three minutes after each mixing. The iron solution is added until there has been no change in the per cent transmission after three successive readings. The data are plotted on graph paper and the point of intersection of the two slopes is taken as the amount of iron necessary to saturate the iron-binding protein (Figure 1). Serum iron determinations were made according to the method of Kitzes, Elvehjem, and Schuette (9). It is possible to determine the total capacity of each sample of serum by totalling the serum iron and the unsaturated binding capacity.

The blood is drawn in the morning with the patient in the fasting state. Lipemic serum, severe icterus, and serum over 24 hours old, were found unsatisfactory. All glassware is carefully cleaned with concentrated nitric acid and glass-redistilled water to render it iron-free. All reagents used are likewise iron-free. Figure 2 shows

⁵ Iron standard is prepared by diluting 14 mgs. of ferrous ammonium sulfate plus 0.5 cc. of 1 N acetic acid to 100 cc. This represents 20 gamma of iron per cc. of standard. Each standard solution is checked by direct iron analysis. Therefore, each addition of 0.05 cc. to 2 cc. of plasma represents an increment of 50 gamma per 100 cc. serum.

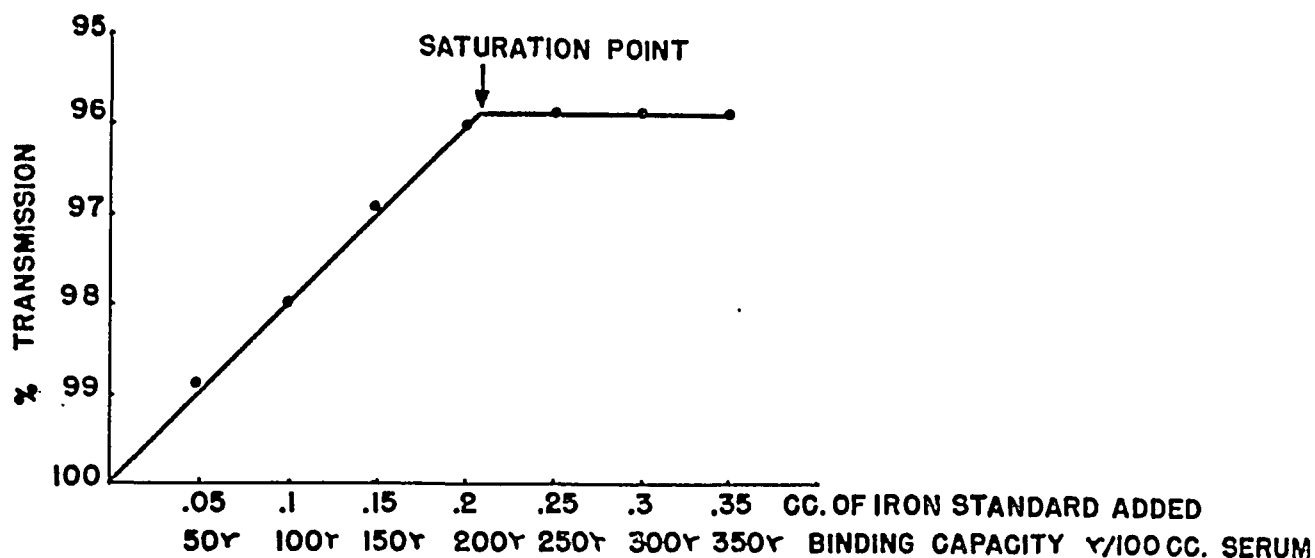


FIG. 1. DETERMINATION OF THE IRON-BINDING CAPACITY OF SERUM

the iron-binding capacity titration with known increments of crystalline iron-binding protein. Table I shows the measured as compared with the calculated increase in iron-binding capacity of serum upon the addition of increments of crystalline iron-binding protein. This method is readily adapted to a colorimeter with a filter of 525 $m\mu$.

RESULTS

Measurements of serum iron, unsaturated iron-binding capacity, total capacity and per cent satu-

ration on 30 normal subjects and 105 patients are shown in Table II, and the groups of particular interest are portrayed graphically in Figure 3. There was no significant difference between men and women. In the combined normal group, serum iron averaged 100 gamma, iron-binding capacity 200 gamma, and total capacity 300 gamma per 100 cc. of serum. Circulating iron-binding protein was 34 per cent saturated with iron. In iron

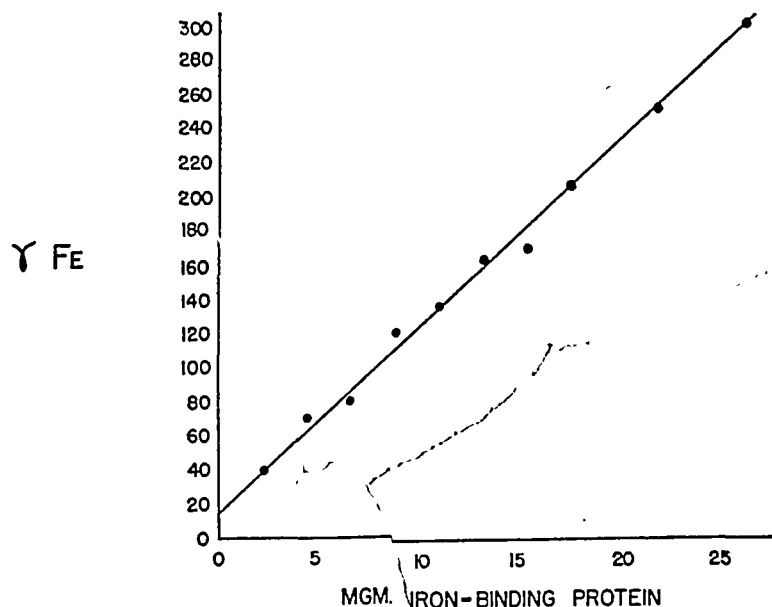


FIG. 2. IRON-BINDING PROTEIN TITRATION

The amount of iron required to saturate increments of the pure β_1 globulin was determined colorimetrically. Each point represents a single determination of iron-binding capacity. Each milligram of protein binds about 1.25 gamma of iron and the measurement is accurate within 25 gamma per 100 cc. When increments of β_1 globulin are added to plasma the same amounts are obtained with an accuracy of within 50 gamma per 100 cc.

MEASUREMENT OF IRON-BINDING CAPACITY OF SERUM IN MAN

TABLE I

Measurement of iron-binding capacity on addition of iron-binding protein to serum

Capacity per 100 cc. of serum	Iron-binding capacity per 100 cc. of crystalline protein added	Iron-binding capacity per 100 cc.	
		Anticipated	Found
gamma/100 cc.	gamma/100 cc.	gamma/100 cc.	gamma/100 cc.
I 100	30	130	140
100	60	160	170
100	90	190	180
100	120	220	235
100	150	250	265
100	180	280	275
100	210	310	300
100	240	340	350
II 250	75	325	330
250	150	400	400
250	225	475	455
250	300	650	630
250	375	725	720

In the table above, increments of crystalline protein of known iron-binding capacity were added to serum with a measured capacity of 100 (I) and to serum with a measured capacity of 250 (II). The increased iron-binding capacity of the serum as determined by the method herein described is compared with the anticipated new binding capacity representing the sum of the capacity of the serum plus the capacity of the added crystalline protein.

deficiency, while the serum iron was lowered, there was an increase above normal in both the unsaturated iron-binding capacity and total carrying capacity of the serum. This is to be contrasted with infection in which serum iron was similarly reduced but where the iron-binding capacity and total capacity were reduced as well. It is of interest that the saturation was below 10 per cent in eight out of ten cases of iron deficiency, while it was above 10 per cent in all ten cases of infection. In general, in patients with iron deficiency who also had carcinoma or other debilitating disease, the per cent saturation was still in the vicinity of 10 per cent, but the total capacity was not significantly increased. In a variety of conditions having in common only general debility and reduction in circulating plasma protein (cancer, infection, liver disease, and renal disease), there was a reduction in the iron-carrying capacity of the serum. Ten normal pregnant women showed no significant deviation from the normal during the first, second, or third trimester of pregnancy. A high serum iron and high percentage saturation of the iron-binding protein were found in refractory anemia, pernicious anemia, hemochromatosis, transfusion hemosiderosis, and liver disease.

Preliminary work has been carried out with Fraction IV-7 processed from human plasma by Surgenor and his associates (8). This material bound *in vitro* 1 mg. of iron per milligram of protein. It has been administered intravenously to 22 individuals in amounts of 2.5-5.0 gms. over periods of 15 to 30 minutes. In two patients, the injection was repeated after two weeks and in neither case was there any reaction. In general, the injections produced a slight rise in serum iron during the first four to six hours, but the maximum rise occurred after a latent period of 12 to 24 hours after injection. The increase in serum iron was as much as 115 gamma per 100 cc. of plasma. This fell over the following two to six days un-

TABLE II

Measurements of serum iron, unsaturated iron-binding capacity, total capacity and per cent saturation on normals and patients

	S. I.	I. B. C.	Total	% Sat.	Hct.
	gamma/100 cc.	gamma/100 cc.	gamma/100 cc.		
Normal Male	147	285	432	34	48
	119	220	339	35	48
	98	200	298	33	40
	88	200	288	30	47
	115	190	305	38	47
	97	220	317	31	48
	107	200	307	35	47
	94	200	294	32	48
	87	200	287	33	43
	136	215	351	39	46
	97	222	319	30	51
	98	222	320	30	47
	104	150	254	41	45
	87	200	287	30	43
	121	150	271	44	46
Average	106	205	311	34	
Normal Female	110	190	300	37	45
	108	150	258	42	45
	120	280	400	30	40
	87	150	237	37	45
	85	250	335	25	45
	95	322	415	22	41
	130	165	295	44	43
	93	165	258	36	43
	84	144	228	37	41
	118	210	328	36	46
	76	148	224	34	40
	72	194	266	37	43
	74	200	274	27	41
	76	200	276	28	38
	82	150	232	35	40
Average	94	194	288	33	
Combined Normal Male and Female	100	200	300	34	

TABLE II—Continued

	S. I.	I. B. C.	Total	% Sat.	Hct.
	<i>gamma/</i> <i>100 cc.</i>	<i>gamma/</i> <i>100 cc.</i>	<i>gamma/</i> <i>100 cc.</i>		
Iron Deficiency Anemia					
Bleeding ulcer	37	395	410	9	29
Bleeding ulcer	60	210	270	22	37
Bleeding hemorrhoids	32	350	382	8	39
Pseudo hemophilia	26	145	171	15	35
Microcytic hypochromic anemia	27	475	502	5	27
Microcytic hypochromic anemia	11	325	336	3	18
Microcytic hypochromic anemia	15	320	345	4	25
Microcytic hypochromic anemia	29	330	359	8	31
Microcytic hypochromic anemia	32	320	352	9	36
Microcytic hypochromic anemia	22	320	340	6	19
Average	29	319	346	9	
Pernicious Anemia					
	129	165	294	44	20
	136	100	236	58	14
	160	50	210	76	18
	47	195	242	19	20
	164	100	264	62	23
	127	57	184	69	14
	136	60	196	69	19
Average	128	104	232	56	
Lymphoma and Leukemia					
Hodgkins	39	185	224	17	44
Lymphosarcoma	82	240	322	25	42
Chronic myelogenous leukemia	49	200	249	20	37
Chronic myelogenous leukemia	90	150	240	37	31
Subacute leukemia	64	61	125	51	23
Aleukemic leukemia	131	40	171	77	30
Acute monocytic leukemia	320	154	474	67	25
Hodgkins	46	169	215	21	29
Agnogenic myeloid metaplasia	80	100	180	44	25
Uremia					
	65	190	255	25	10
	77	110	187	41	20
	54	100	154	35	
	67	180	247	27	29
	35	150	185	19	20
	47	299	346	14	34
	50	157	207	24	27
	36	258	294		22
Transfusion Hemosiderosis					
	297	0	297	100	54
	305	0	305	100	30
	236	0	236	100	45
	207	0	207	100	11
Average	260	0	260	100	

TABLE II—Continued

	S. I.	I. B. C.	Total	% Sat.	Hct.
	<i>gamma/</i> <i>100 cc.</i>	<i>gamma/</i> <i>100 cc.</i>	<i>gamma/</i> <i>100 cc.</i>		
Infection					
Pneumonia	117	100	217	54	42
Pyrexia of unknown origin	27	170	197	14	29
Subacute bacterial endocarditis	26	220	246	11	29
Chronic pulmonary infection	21	140	161	13	54
Trichinosis	64	180	244	26	34
Subacute bacterial endocarditis	37	185	222	17	42
Pelvic inflammatory disease	42	165	207	20	41
Miliary tuberculosis	40	210	250	16	42
" "	41	195	236	17	48
Pyrexia of unknown origin	32	195	227	16	36
Average	44	176	220	20	
Hemochromatosis					
	233	0	233	100	
	170	50	220	77	
	235	50	285	82	48
	245	0	245	100	47
	250	57	307	82	42
	250	0	250	100	44
	204	0	204	100	45
	204	0	204	100	46
	220	50	270	81	47
Average	224	23	247	91	
Liver Disease					
Acute hepatitis	121	150	270	45	35
Cirrhosis	137	175	312	44	48
" "	140	160	300	47	25
Cirrhosis with hepatitis	100	50	150	66	35
Cirrhosis	65	150	215	30	33
" "	142	57	199	71	35
Cirrhosis with hepatitis	85	150	235	36	32
Cirrhosis	121	100	221	55	40
Cirrhosis, terminal	91	55	146	62	47
Average	111	116	227	50	

less the patient had hemosiderosis or hemochromatosis, in which case the elevation was maintained over a longer period. A second injection of globulin given to the same patient did not produce a rise in serum iron level except in cases of iron excess.

DISCUSSION

The validity of this measurement of the iron-binding protein capacity of human sera would seem to be established in a number of ways: (1) the increased capacity produced by the addition of

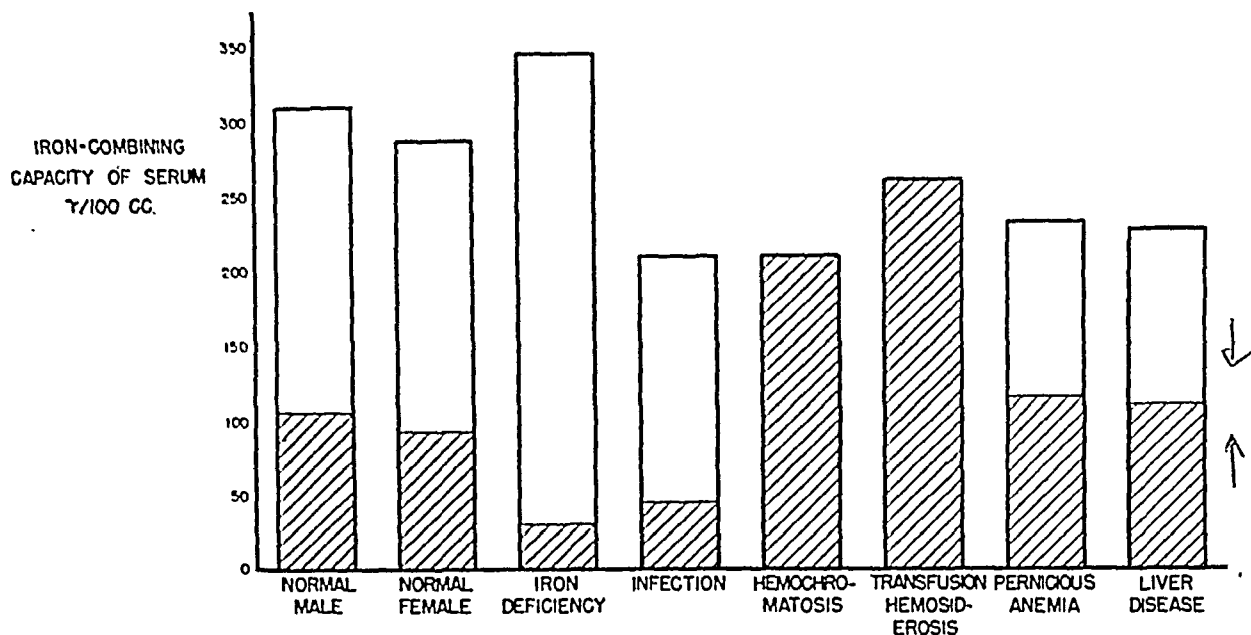


FIG. 3. IRON-BINDING CAPACITY OF SERA

The iron-binding capacity of human serum is represented in block diagram. The cross-hatched portion represents serum iron, and the clear area the unsaturated capacity of the iron-binding protein in gamma per 100 cc.

known amounts *in vitro* of pure iron-binding globulin to plasma may be measured with an error of less than 50 gamma per 100 cc.; (2) the intravenous injection of iron-binding protein results in an increase in the measured binding capacity proportionate to the amount of protein given; (3) injections of iron or of nonviable erythrocytes will result in increase in serum iron to the point of total binding capacity as previously measured but not beyond it; (4) those patients with saturation of their iron-binding protein show no significant increase in serum iron after oral or intravenous iron administration.

The observation that serum iron cannot be increased above the measured saturation point of the globulin confirms other evidence that iron cannot exist in a free state in the serum. The only exceptions to this are the injections of massive amounts of iron ascorbate (10) or iron ascorbate gelatin complexes and the serum of terminal hemochromatotic patients where very high levels of serum iron may be found. It would appear in both of these instances that the iron is bound to some other protein complexes. This complete protein binding of iron in the body, whether intracellular

or within the blood stream, probably explains the inability of the body actively to excrete iron.

Holmberg and Laurell (11) have reported similar measurements of the iron-binding capacity of serum, employing a different method dependent on the color reaction between dipyrldyl and unbound iron. In normal subjects the serum iron averaged 130 gamma per 100 cc. and the total capacity was 312 gamma per 100 cc. These figures are in good agreement with our data. As pointed out by these authors, the increase in serum iron and the maximum level attained following oral and intravenous iron tolerance tests are limited by the amount of iron-binding globulin in circulation. For example, the initial height of 291 gamma per 100 cc. observed by Waldenstrom (12) following intravenous injection of iron in normal subjects was very close to the total capacity as measured by Holmberg and Laurell and by us. The lower average level obtained by Wintrobe of 168 gamma per 100 cc. (13), in patients with infection after intravenous iron, again is consistent with the reduced binding capacity found in our group with infection.

The lack of response of serum iron in patients

with untreated pernicious anemia or hemochromatosis to oral iron administration is considered to be due to the pre-existing high degree of saturation of the β_1 globulin which will allow little or no increase in serum iron. The unaltered level of serum iron does not, however, preclude iron absorption, since the serum iron level is not an expression of the turnover rate of iron in the serum. This will be the subject of a later report.

The total binding capacity of normal sera of about 300 gamma per 100 cc. represents about 250 mgs. of iron-binding protein per 100 cc. of plasma.⁶ Increases in iron-binding protein were observed by us in iron deficiency, and have been reported by Laurell (14) in pregnancy. These increases parallel the need of the body for more efficient iron absorption and transport. Further work is necessary to determine whether this increase in iron-binding protein is responsible for the increased iron absorption in these conditions. In other conditions decreases in total binding capacity to 50 per cent of normal have been observed, but it seems improbable that this reduction in any instance was capable of significantly impairing iron transport since there was an appreciable amount of unsaturated iron-binding protein still in circulation.

It would appear that the level of serum iron and the per cent saturation of the iron-binding protein are carefully regulated under normal circumstances. Conditions in which saturation of iron-binding protein is increased are those involving bone marrow block, iron excess, and severe liver disease. The important role of the liver in serum iron regulation is not unexpected since this organ is the chief iron storage depot of the body. Whether or not conditions of iron excess may be recognized without some degree of hepatic dysfunction is not yet clear. At any rate, it has been possible to make the diagnosis in nine cases of hemochromatosis by this technique and to separate these cases from simple cirrhosis. Depression of the per cent saturation occurs in iron deficiency and in infections. It seems reasonable to explain the former on the basis of depletion of body iron and the latter on an increased affinity of tissue storage depots for iron (15).

Laurell (14) in a very comprehensive and excellent study of iron transport has repeated meas-

urements of the iron-binding protein in a variety of conditions. Although different methods were used, the results we have obtained are in perfect agreement with those of Laurell. We hesitate to accept the hypothesis that the degree of saturation may regulate iron transport and iron absorption. For example, in animals on diets which allow excessive iron absorption, the serum-binding protein becomes completely saturated with iron after about two weeks, yet iron absorption continues fully as rapidly over the following three to four weeks (16). Movement of iron within the body may be managed by the respective affinity of various tissues for iron, in which system the carrier protein of the serum would play a passive role. Injections of iron-binding protein exert only a very temporary effect on the serum iron level. It remains to be determined how much one may aid or interfere with iron transport by increasing or decreasing the amount of iron-binding protein.

SUMMARY

A method is described for the measurement of the iron-binding capacity of human serum. Measurements of serum iron, total iron-binding capacity, and per cent saturation of this protein are reported in normal subjects and in a variety of diseases. The implications of these findings are discussed.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Doctor Cohn and his associates for the materials used in part of this study.

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⁶ One milligram β_1 globulin binds about 1.25 gamma of tissue as demonstrated in Figure 1.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE
PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXIX.
THE ANEMIA OF INFECTION. STUDIES ON THE
IRON-BINDING CAPACITY OF SERUM¹

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It has been generally recognized since Barkan's original observations in 1929 (1) that the iron in serum is non-dialyzable and non-ultrafilterable at pH 7.3 and, therefore, is probably protein-bound (2-4). To which of the serum proteins the iron is bound was not clearly established until recently. Earlier work indicated that half-saturation with ammonium sulfate precipitated serum iron quantitatively together with the globulins (5). Vahlquist (4) with the aid of the electrophoretic technique concluded that the iron in serum is bound to both albumin and globulin, the α and β globulins serving as the principal carriers.

Following their demonstration of a protein component in raw egg white capable of binding iron (6), Schade and Caroline investigated various fractions of human plasma prepared by E. J. Cohn and his associates for a similar property (7) and discovered that, qualitatively, Fraction IV-3, 4 was active in this respect, as shown by biological and colorimetric tests. By colorimetry it was found that 1 mg. of protein took up a maximum of 0.44 μ g of Fe^{++} . Subfractionation of Fraction IV-3, 4 revealed that the iron-binding protein resides almost completely in a β_1 globulin (Fraction IV-7) (8). This fraction constitutes approximately 3 per cent of the total plasma protein.² Two molecules of either Fe^{++} or Fe^{+++} are bound to one of protein. If the molecular weight of the β_1 globulin is 90,000, the iron content of the iron-protein complex is 0.125 per cent. Studies over a wide range of pH have revealed that at neutral pH the iron is non-dialyzable whereas at pH 5 the iron becomes dialyzable.

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² Cohn, E. J., Personal communication.

Skouge (9) and Waldenström (10) have pointed out that the concentration of serum iron five minutes after the intravenous injection of iron is lower than would be expected on the basis of the quantity injected. This "braking" phenomenon was more pronounced in patients with pernicious anemia in relapse than in normal persons. When the initial serum iron was high, scarcely any rise was noted after the intravenous injection of iron. Furthermore, toxic reactions were observed long before the generally tolerated dose of 10 mg. had been administered. Holmberg and Laurell (11) by means of a colorimetric method in which α - α' -dipyridyl was used, were able to study the capacity of serum to bind iron *in vitro*. They found the "saturation limit" in 10 normal subjects to be 312 μ g per cent (range 264 to 366 μ g). The saturation values corresponded well with the maximum values of approximately 291 μ g per cent obtained by Waldenström (10) after the intravenous injection of 10 mg. of Fe^{++} . These authors explain the "braking effect" observed by Waldenström by assuming that the unbound iron is rapidly eliminated from the circulation. Toxic effects after the intravenous administration of iron were noted only when the saturation limit was exceeded and surplus iron left the blood stream.

Laurell (12) has recently published a comprehensive monograph on the iron-binding component in human plasma. In 100 normal subjects the mean value for the iron-binding capacity of the serum was 315 ± 3.3 μ g per cent. In pregnancy, infectious hepatitis, and acute and chronic blood loss the capacity of the serum to bind iron was found to be increased. In acute and chronic infections, pernicious anemia, hemolytic anemia, cirrhosis of the liver, uremia and malignancy the capacity was found to be diminished. On the basis of these results Laurell has advanced the hypothesis that the body is capable of mobilizing iron from

the depots and, at the same time, can increase the absorption of iron by either lowering the serum iron concentration or by increasing the iron-binding component of the serum. Conversely, by either increasing the serum iron or lowering the iron-binding protein component more iron can be transferred to the depots and the absorption of iron checked.

Studies in this laboratory dealing with the pathogenesis of the anemia of infection have revealed that in the presence of infection a disturbance in iron metabolism occurs (13, 14). There is a pronounced and persistent hypoferremia and when iron is injected intravenously the magnitude of the subsequent increase in plasma iron is less than in normal individuals. The rate of disappearance of iron from plasma following injection has been found to be twice that seen in normal subjects. Neither by repeated intravenous injections nor by the continuous intravenous infusion of iron has it been possible to increase the iron level beyond a certain point. A "braking" mechanism thus operates in the presence of infection which is so powerful that as more iron is given it becomes increasingly difficult to maintain the plasma iron concentration.

In view of these observations it was thought desirable to investigate the role of the iron-binding protein of the serum in the pathogenesis of the anemia of infection. The purpose of this paper is to describe *in vitro* and *in vivo* studies of the iron-binding capacity of serum in normal subjects, in patients with infection, and in animals in which infections were produced experimentally.

METHODS

Preparation of serum. From fasting subjects an amount of venous blood sufficient to provide 8 to 10 ml. of serum was drawn without hemolysis into a syringe and transferred to a centrifuge tube. The blood was allowed to clot, the latter was separated from the glass and the blood was then centrifuged at about 2500 r.p.m. for 15 minutes. The serum was then transferred to a second centrifuge tube and respun to remove all red cells. All determinations were made within three hours after the blood was collected.

Serum iron (SI). For the determination of serum iron the method of Barkan and Walker (15) using the Evelyn photoelectric colorimeter was followed except that twice the quantities recommended by them were used. This required 4 ml. of serum. The results are expressed in μg per cent.

Unsaturated iron-binding capacity (UIBC). For the determination of the unsaturated capacity to unite with

iron, i.e., the binding capacity in addition to that which is already fixed (SI), a modification of the method of Schade and Caroline (7) was used. This method is based on the principle that the iron-protein compound is salmon pink in color (maximum absorption at about 460 $m\mu$) and that the color development is proportional to the amount of iron-protein formed. The serum is therefore titrated with iron until the maximum color develops. The results are expressed in terms of μg of iron bound.

Two ml. of serum and 8 ml. of 0.9 per cent saline are added to each of two colorimeter tubes. Using the Evelyn photoelectric colorimeter and the 490 filter, the blank is set at 100 per cent transmission. The per cent transmission of the other tube is then read. One-tenth ml. of glass-distilled water is added to the blank and

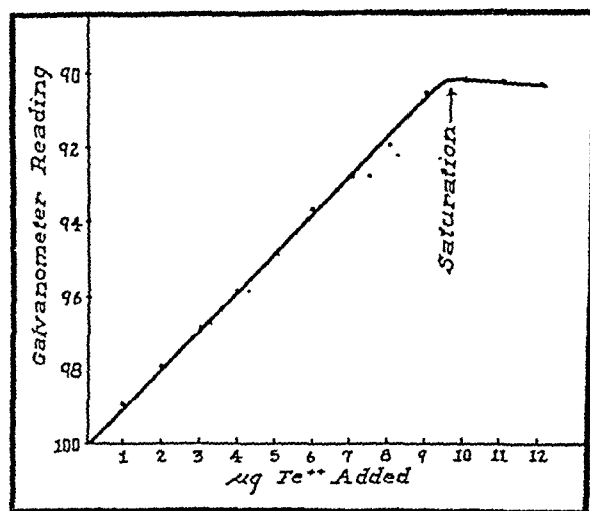


FIG. 1. MEASUREMENT IN HUMAN SERUM OF THE SALMON PINK COLOR DUE TO THE FORMATION OF THE IRON-PROTEIN COMPLEX

Evelyn photoelectric colorimeter. Filter 490.

0.1 ml. of a standard iron solution containing 1 μg of iron is carefully added to the other tube. The tubes are shaken and the per cent transmission is determined four minutes after shaking, again setting the blank at 100. This procedure is repeated until there has been no change in the per cent transmission on three successive readings. The per cent transmission is then plotted against the micrograms of iron added and the saturation point is estimated as illustrated in Figure 1.

The UIBC is calculated by multiplying by 50 the micrograms of iron needed to produce saturation. The values are expressed in μg per cent and represent the micrograms of iron with which 100 ml. of serum are capable of uniting.

Total iron-binding capacity (TIBC). This is calculated by adding the unsaturated iron-binding capacity to the serum iron and is expressed in μg per cent.

Per cent saturation. This is calculated by dividing the serum iron by the total iron-binding capacity.

All glassware must be carefully cleaned with concentrated HCl, washed three times with glass-redistilled water and air dried under cover.

A standard iron solution is prepared by dissolving 0.1 gm. of standard iron wire in 50 ml. of 1:3 nitric acid. The solution is boiled to expel oxides of nitrogen and is then diluted to 1 liter with glass-distilled water. A dilute working standard containing 10 μg of iron per 1 ml. is prepared from this. If properly stored this solution will not change in concentration for several months.

Comment. This method has been found to work equally well when either ferric ammonium sulfate or iron wire is used for the standard solution. The absorption spectrum of the pink iron-protein compound has a rather broad base with a maximum absorption at about 460 $m\mu$. For this reason any filter between 420 and 520 $m\mu$ is suitable but less interference from hemoglobin and bile pigment compounds is encountered in the region of 490 $m\mu$. One to three minutes are required for the development of maximum color after the addition of iron. Fresh, non-lipemic, non-icteric serum must be used. If sufficient serum is not available for a blank this may be dispensed with although the accuracy of the method is thereby slightly reduced. The concentration of the chromogen is low and great care must be taken in making the readings.

Since increments of 1 μg iron are added to 2 ml. of serum and the results are multiplied by 50 in order that they may be expressed in μg per cent the method can be no more accurate than 50 μg per cent. However, triplicate determinations on the same serum have repeatedly given the same result.

EXPERIMENTAL

To determine whether the *in vitro* measurement of the TIBC of the serum is a true measure of the ability of the serum to combine with iron *in vivo*, an amount of iron calculated to exceed the determined TIBC was injected intravenously into each of eight individuals. The iron was administered in the form of a 0.5 per cent solution of iron ascorbate in amounts equivalent to 0.137 mg. Fe^{++} per kilogram of body weight. An initial sample of blood was withdrawn, the iron solution injected in 30 to 60 seconds, and five minutes later a second sample was taken from the other arm. The results are presented in Figure 2. In each instance

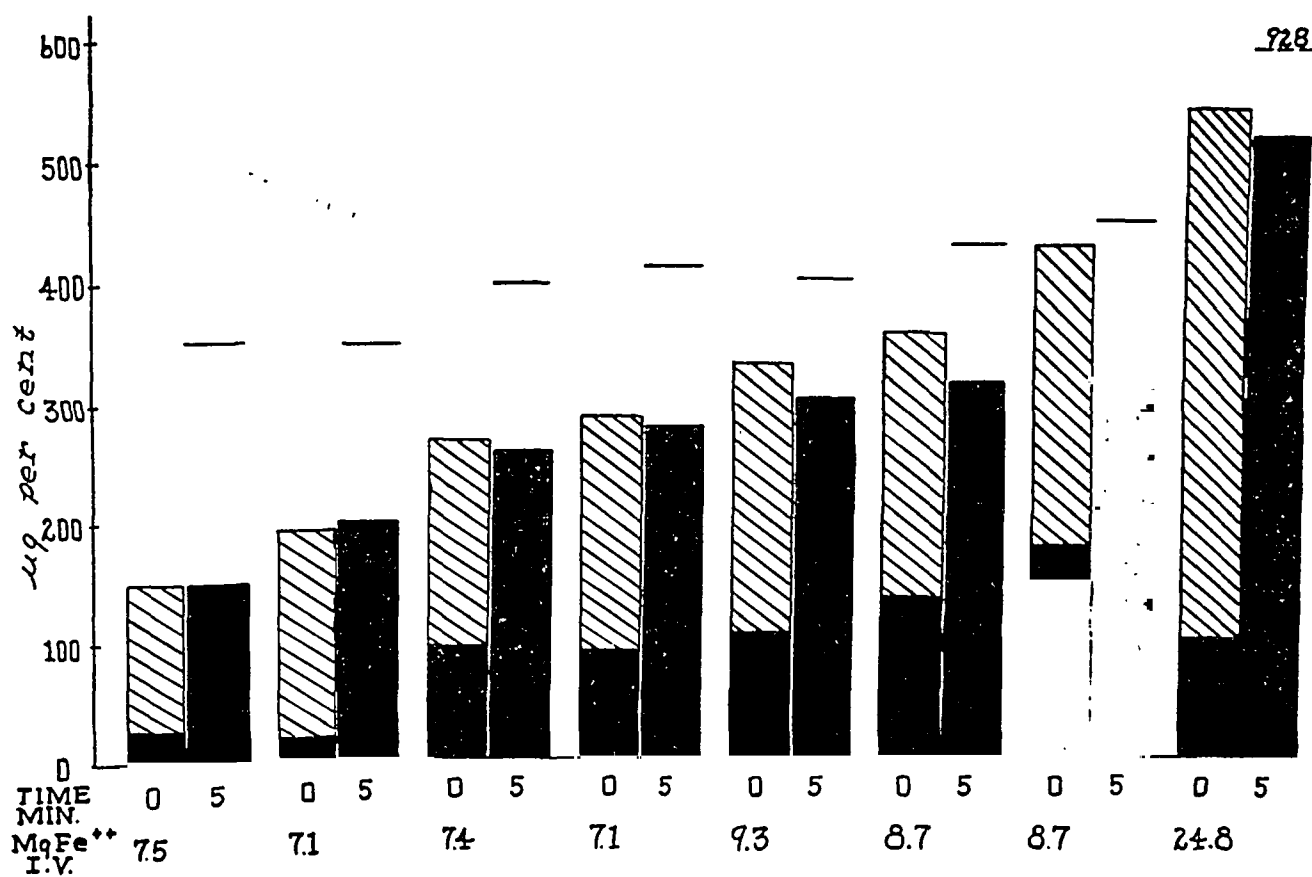


FIG. 2. COMPARISON OF THE SERUM IRON LEVEL, THE UNSATURATED PROTEIN AND THE TOTAL IRON-BINDING CAPACITY BEFORE AND AFTER THE INTRAVENOUS INJECTION OF IRON

The horizontal lines represent the expected serum iron values as calculated from the amount of iron administered and the estimated plasma volume. Solid areas represent the serum iron; hatched areas represent the unsaturated capacity to bind iron. The total height of each column represents the total iron-binding capacity. The amount of iron (Fe^{++}) injected is indicated below the various columns.

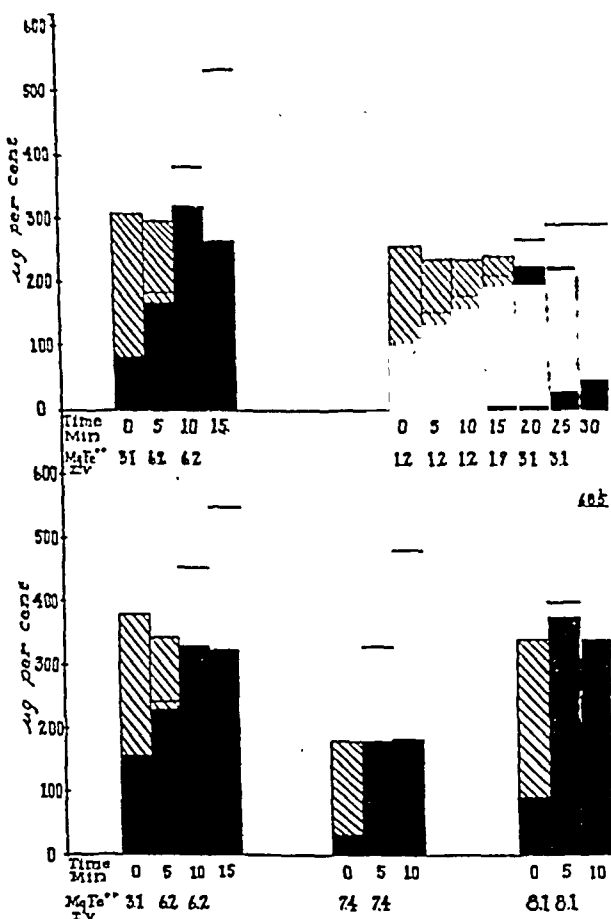


FIG. 3. RESULTS OF THE REPEATED INTRAVENOUS INJECTION OF IRON AT FIVE-MINUTE INTERVALS IN EACH OF FIVE PATIENTS

The horizontal lines represent the expected serum iron values on the basis of the amount of iron administered and the calculated plasma volume. Solid areas represent the serum iron. Hatched areas represent the unsaturated iron-binding capacity. The total height of each column represents the total iron-binding capacity. The amount of iron (Fe^{++}) injected is indicated below the various columns.

the serum was completely saturated with iron ($\text{UIBC} = 0$) and the measured amount of serum iron corresponded, within the limits of the experimental error, to the previously determined TIBC. A reaction to the injection, manifested by headache, flushing and nausea, was noted within two minutes in each individual. By the time the second specimens were withdrawn the reactions had completely disappeared.

The results of the repeated injection of iron at five-minute intervals into each of five patients are presented in Figure 3. After complete saturation

had been attained the loss of additional iron from the serum within five minutes was approximately 100 per cent. Repeated injections of iron failed to raise the serum iron level above the TIBC. However, when amounts insufficient to reach the saturation limit were given, not all of the iron was retained in the serum, only 50 to 80 per cent being accounted for when plasma volume was calculated as equivalent to 43.1 ml. per kilogram of body weight for males, and 41.5 ml. per kilogram for females. Since it is unlikely that the calculated plasma volume would be in error to the extent of 50 per cent, it is possible that in some instances iron escaped to the tissues even though the serum possessed the capacity to bind it. Toxic reactions were observed only when the TIBC was exceeded.

Determinations of the TIBC have been made in 15 normal males and 15 normal females. The results are presented in Table I. The mean \pm S.D. for the entire group was 359 ± 30.8 μg per cent and the range 306 to 429 μg per cent. The values for the female group were somewhat higher than for the male group. In these normal subjects 35 ± 6.4 per cent (26 to 49) of the total iron-binding capacity was saturated.

Studies have been made on 13 patients with chronic infections and six patients with iron deficiency anemia. The results are presented in Figure 4. In chronic infections, in each instance, the TIBC as well as the serum iron was reduced below the lower limits of normal. The reduction in serum iron was proportionately greater than the decrease in TIBC, the per cent saturation being

TABLE I

Summary of the normal data

Group	No. determ.	UIBC	SI	TIBC	% Sat.
Males	15	220 ± 24.7 (180-250)	127 ± 29.2 (79-196)	347 ± 26.1 (306-396)	36 ± 7.78 (24-49)
Females	15	248 ± 34.4 (200-300)	123 ± 19.2 (101-164)	371 ± 35.9 (316-429)	33 ± 4.92 (26-42)
Total	30	234 ± 29.4 (180-300)	125 ± 24.2 (79-196)	359 ± 30.8 (306-429)	35 ± 6.4 (26-49)

UIBC refers to unsaturated iron-binding capacity in μg per cent.

SI refers to serum iron in μg per cent.

TIBC refers to total iron-binding capacity in μg per cent.

% Sat. (per cent saturation) = $\frac{\text{SI}}{\text{TIBC}} \times 100$

Figures represent mean \pm standard deviation.

Figures in parenthesis represent range.

between 7 and 26 per cent in all but two patients. In these two cases the decrease in serum iron was proportional to the decrease in TIBC and the per cent saturation remained within the limits of normal (26 to 49 per cent). In contrast, in the patients with iron deficiency anemia the TIBC was increased above 450 μg per cent. Since the serum iron is very low in iron deficiency, the per cent saturation was below 10 in all.

The change in TIBC in relation to the rise in serum iron which occurs during recovery from infections was followed in six patients. The results are presented in Figures 5-9, and 12. These studies showed that in infections the total iron-binding capacity per 100 ml. serum increased, as did the serum iron, following successful treatment and anemia, when present, tended to disappear (Figures 5-7). The per cent saturation of the iron-binding protein with iron did not change greatly.

In a more chronic infection (Figure 8) the changes following streptomycin therapy were very gradual but in another case (lung abscess, Figure 9) the improvement following thoracotomy was more striking.

To determine if the intravenous administration of metal-combining globulin (Fraction IV-7) together with iron might increase the TIBC to normal and thereby prevent the rapid disappearance of iron from the serum which occurs in the presence of infection, these substances were given to two patients with chronic infections. In each patient the rate of disappearance of iron from the serum after its intravenous injection was measured two days prior to the administration of the metal-combining globulin, sample measurements being taken at 0, five min., one, three, five, seven, 24, 48 and 96 hours after the injection. The amount of globulin to be administered was then added to 500

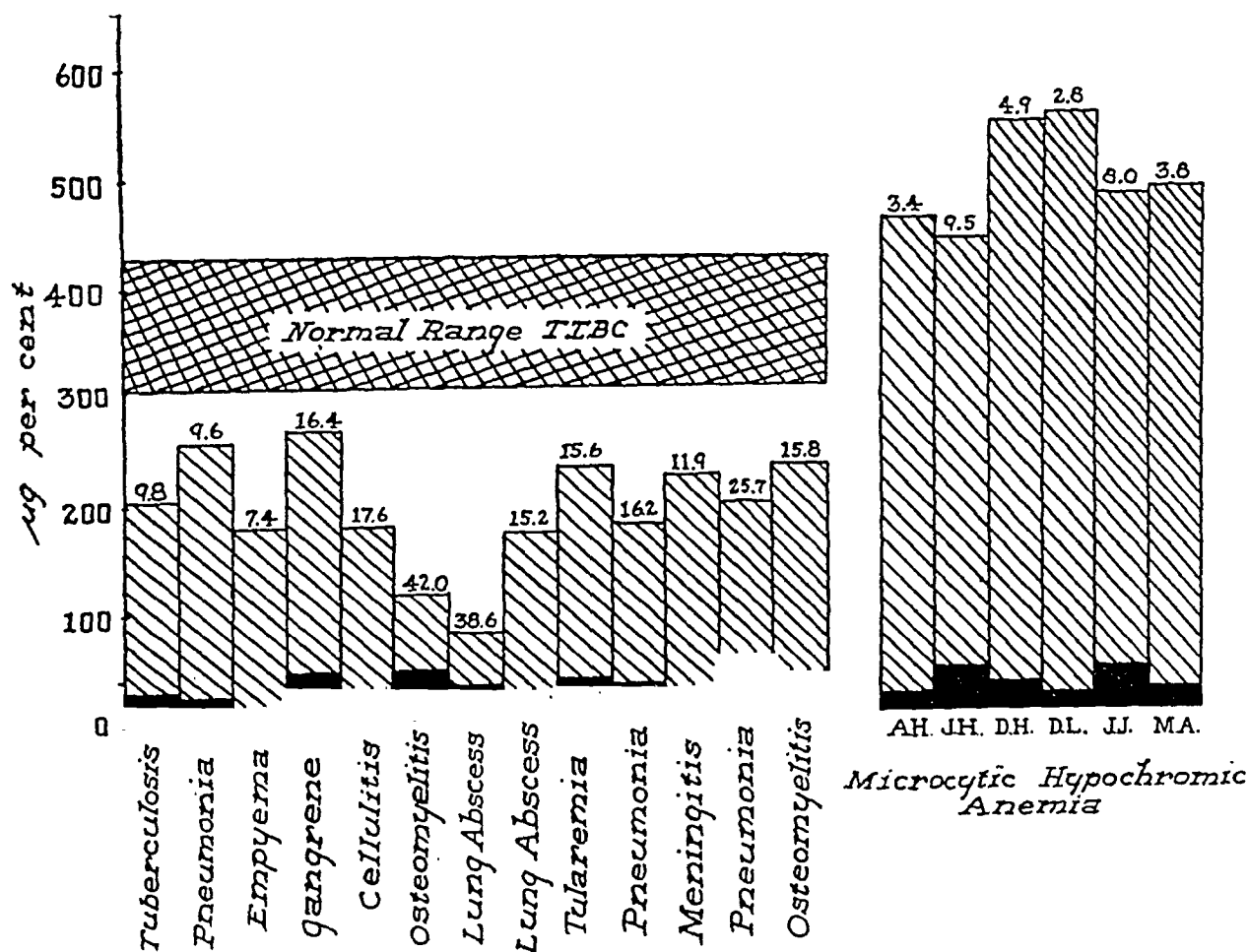


FIG. 4. THE TOTAL IRON-BINDING CAPACITY OF THE SERUM IN 13 CASES OF ANEMIA ASSOCIATED WITH INFECTION AS COMPARED WITH THE NORMAL AND SIX CASES OF IRON DEFICIENCY ANEMIA

Solid areas represent serum iron. Hatched areas represent the unsaturated iron-binding capacity. The total height of each column represents the total iron-binding capacity of the serum. The figures above the columns represent the per cent saturation (SI/TIBC).

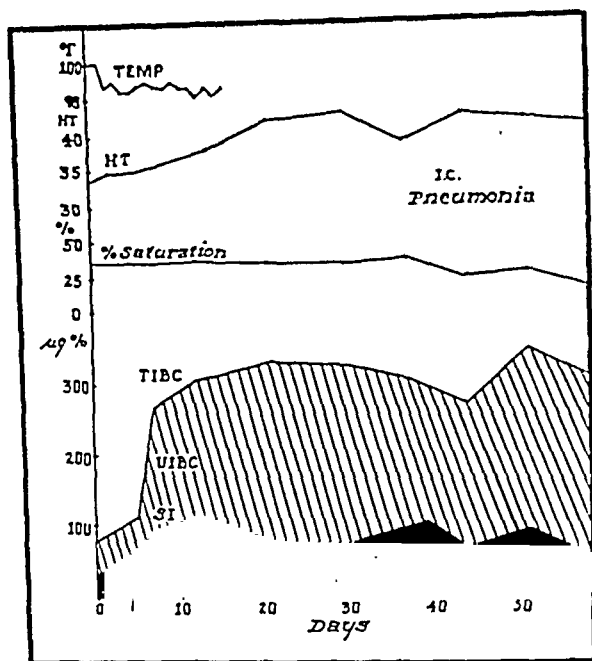


FIG. 5. SHOWING THE SHARP RISE IN THE IRON-BINDING CAPACITY OF THE SERUM (TIBC) IN A PATIENT (I. C.) RECOVERING FROM LOBAR PNEUMONIA, THE INCREASE IN SERUM IRON (SI) AND THE DISAPPEARANCE OF ANEMIA (REPRESENTED BY THE VOLUME OF PACKED RED CELLS (Ht.))

UIBC refers to unsaturated iron-binding capacity. Per cent saturation is the ratio of SI to TIBC.

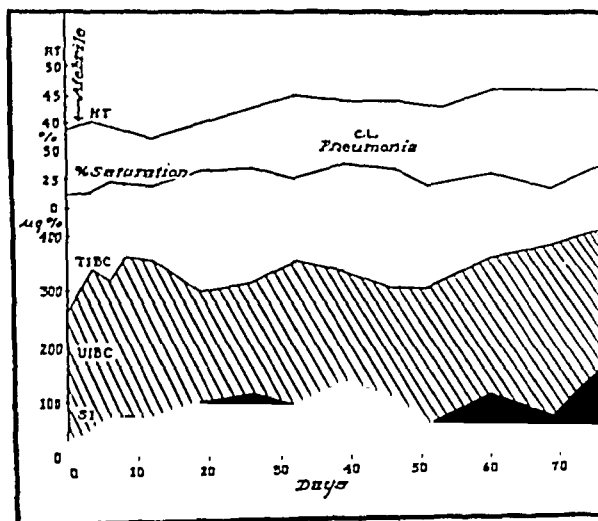


FIG. 6. SHOWING THE RISE IN THE IRON-BINDING CAPACITY OF THE SERUM IN A PATIENT (C. L.) RECOVERING FROM LOBAR PNEUMONIA, THE IRREGULAR INCREASE IN SERUM IRON AND THE DISAPPEARANCE OF ANEMIA
For symbols see Figure 5.

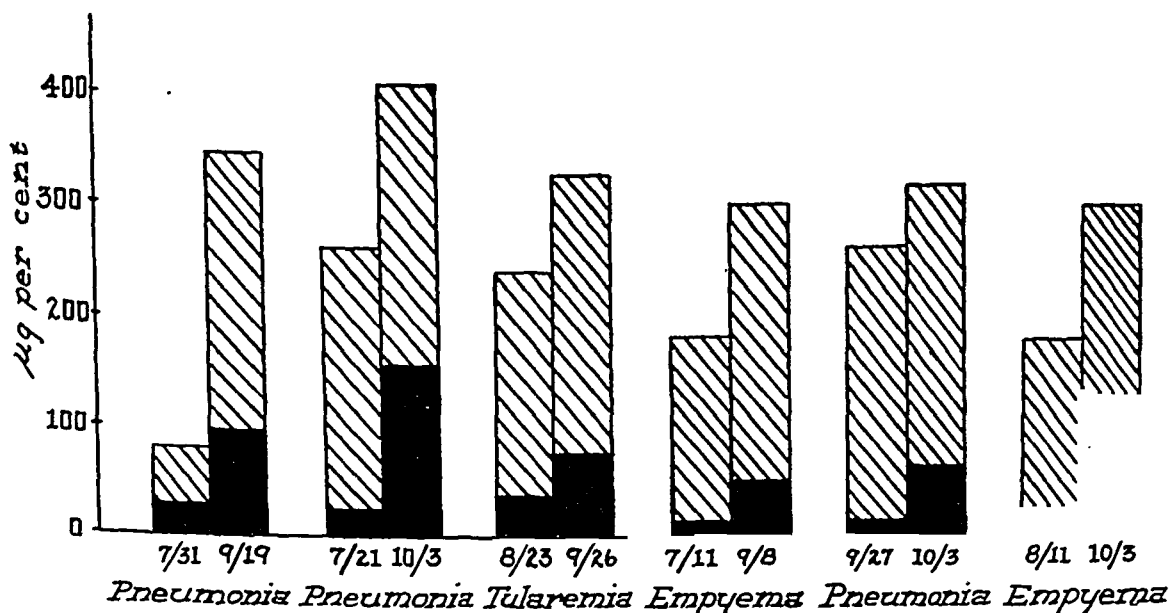


FIG. 7. UNSATURATED IRON-BINDING CAPACITY, SERUM IRON AND TOTAL IRON-BINDING CAPACITY IN SIX CASES DURING THE HEIGHT OF THE ILLNESS AND AFTER RECOVERY
For symbols see Figure 2.

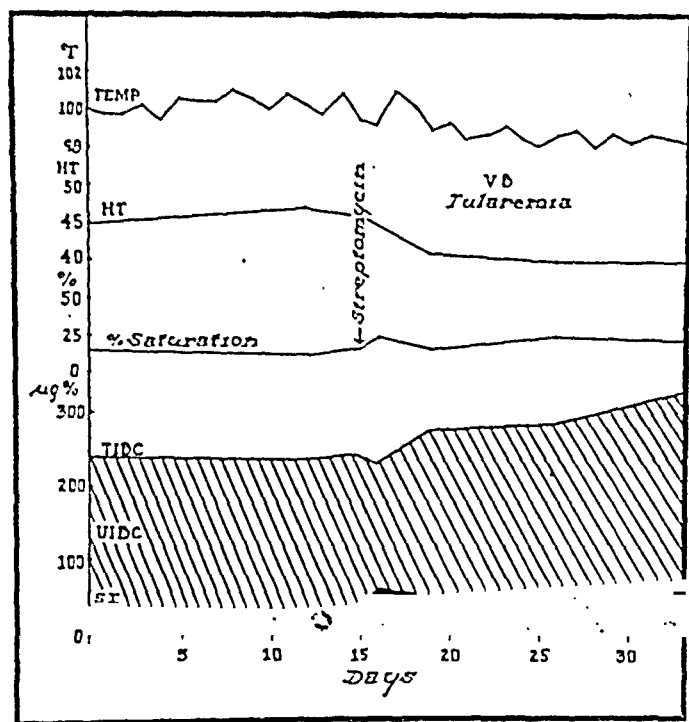


FIG. 8. SHOWING THE SLIGHT RISE IN THE IRON-BINDING CAPACITY OF THE SERUM IN A PATIENT (V. B.) DURING RECOVERY FROM TULAREMIA

For symbols see Figure 5.

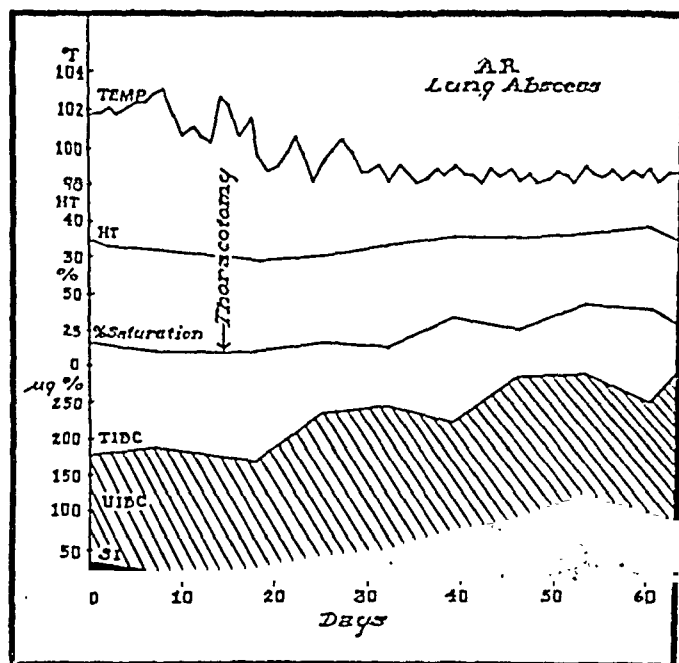


FIG. 9. SHOWING THE RISE IN THE IRON-BINDING CAPACITY OF THE SERUM, THE RISE IN SERUM IRON AND MINIMAL IMPROVEMENT IN ANEMIA IN A PATIENT (A. R.) FOLLOWING THORACOTOMY FOR A LUNG ABSCESS

For symbols see Figure 5.

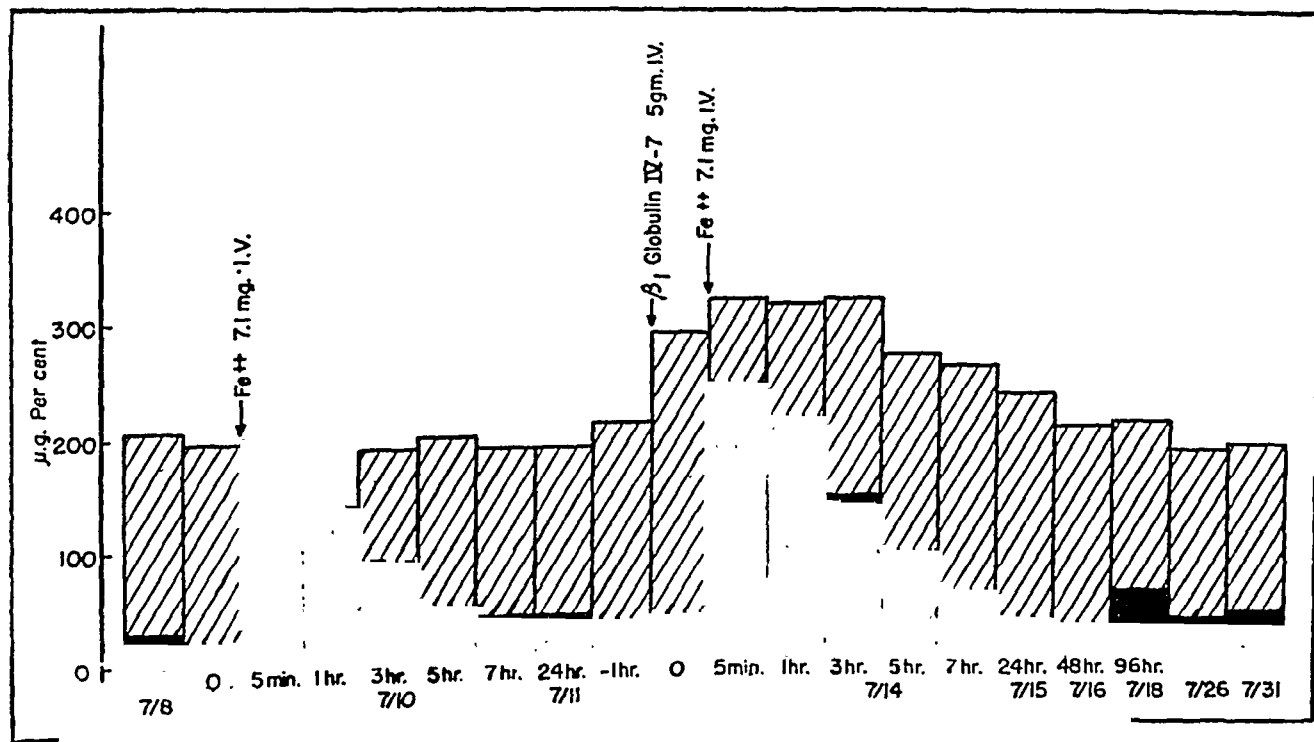


FIG. 10. THE EFFECT OF THE INJECTION OF 5 GM. OF METAL-COMBINING GLOBULIN (FRACTION IV-7) ON THE TOTAL IRON-BINDING CAPACITY OF THE SERUM IN A PATIENT (J. A.) WITH TUBERCULOSIS

For symbols see Figure 2. The serum iron rose to a somewhat higher level following the injection of iron than before globulin was given and the subsequent decrease in serum iron was slower but after 24 hours the level was the same as originally.

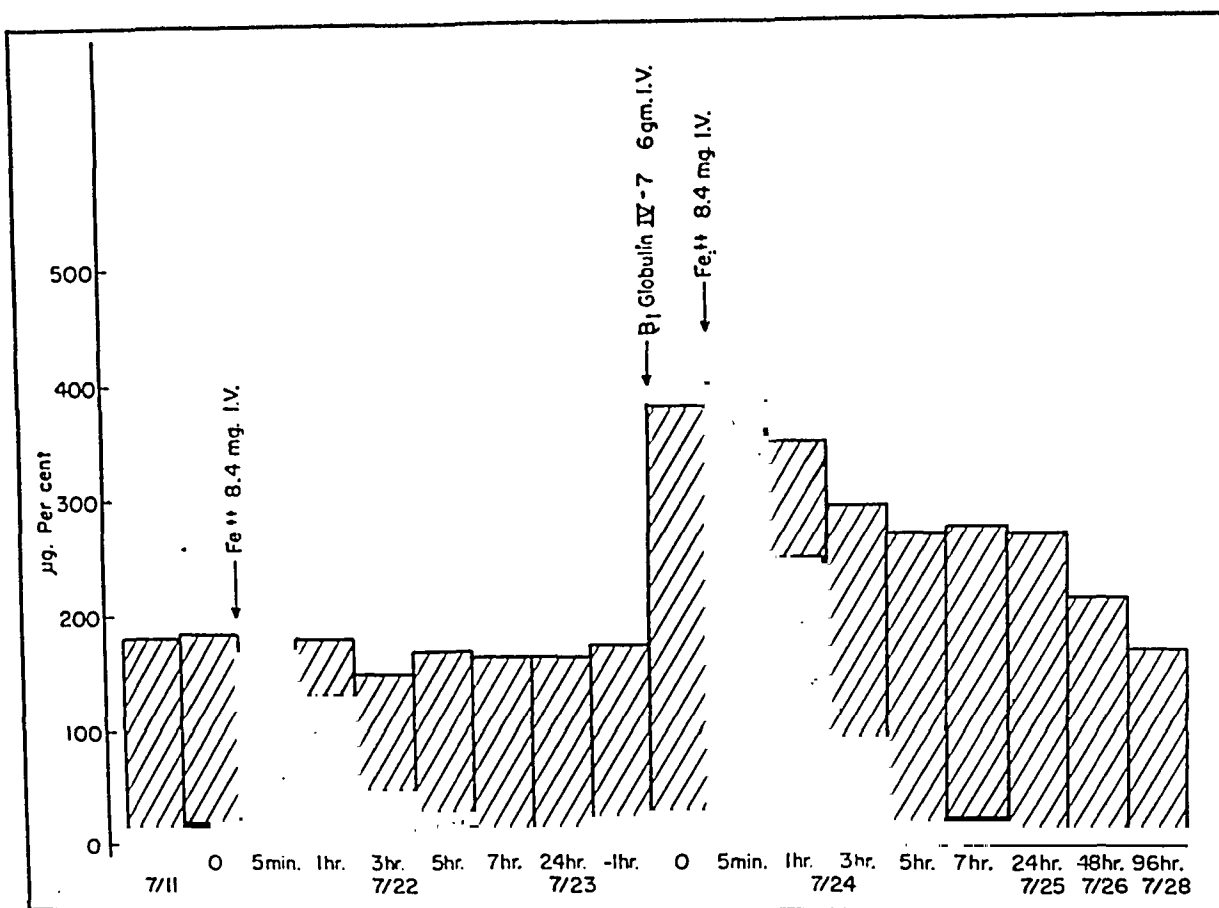


FIG. 11. THE EFFECT OF THE INJECTION OF 6 GM. OF METAL-COMBING GLOBULIN (FRACTION IV-7) ON THE TOTAL IRON-BINDING CAPACITY OF THE SERUM IN A PATIENT (J. K.) WITH CHRONIC EMPYEMA

Although the TIBC increased to a higher level than in patient J. A. (Figure 10) this did not succeed in maintaining an elevated serum iron level. For symbols see Figure 2.

ml. of saline and given over a one-hour period. Five minutes after this was completed a sample of blood was withdrawn for analysis and the same quantity of iron injected (0.137 mg. per kilogram body weight) as had been given two days previously. The results are presented in Figures 10 and 11 and illustrate several points of interest. The administration of the metal-combining globulin increased the TIBC to within normal limits in both patients. The iron-binding capacity of the protein injected was $0.72 \mu\text{g Fe}^{++}$ per milligram of protein. Therefore, all of the material injected could be accounted for in the serum on the basis of the calculated plasma volume. There was no evidence of inactivation of the binding capacity within two to three hours. The levels of serum iron reached when metal-combining globulin was given in addition to iron were somewhat higher in one case

and much greater in the second case, as compared with those obtained when iron was injected alone. The rate of disappearance of iron from the serum was not diminished, however. In both individuals the serum iron returned to its previously low level within seven hours in spite of the increased TIBC. The TIBC returned to its previously low level in 48 hours in one patient (Figure 10) and in 96 hours in the other (Figure 11). The temporary restoration of the TIBC to normal in these cases apparently did not result in a detectable mobilization of iron from the tissues to the blood.

It is of interest to note that while the intravenous injection of metal-combining globulin together with iron was not followed by a significant change in the serum iron, thoracotomy with relief of the underlying disorder was associated with a steady increase in total iron-binding capacity, a gradual

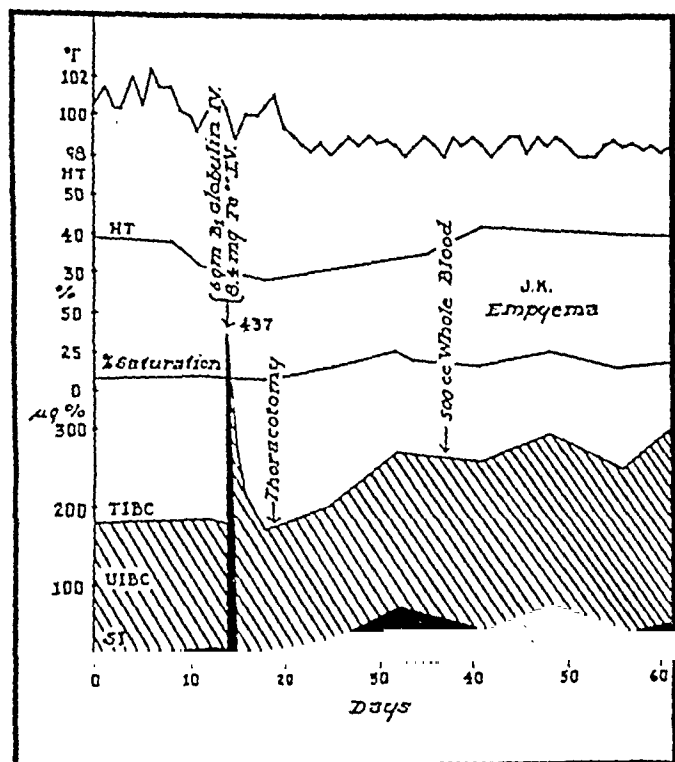


FIG. 12. TO COMPARE THE VERY TRANSIENT RISE IN THE TOTAL IRON-BINDING CAPACITY AND SERUM IRON IN A PATIENT (J. K.) FOLLOWING THE INJECTION OF 6 GM. OF METAL-COMBINING GLOBULIN (FRACTION IV-7) AND 8.4 MG. Fe^{++} AS IRON ASCORBATE WITH THE STEADY RISE FOLLOWING THORACOTOMY WITH RELIEF OF THE EMPYEMA

The serum iron also rose. The saturation of the iron-binding protein with the iron increased and the anemia disappeared. (Same patient as in Figure 11.) For symbols see Figure 5.

rise in serum iron, increased saturation of the iron-binding protein with iron and relief of the anemia (Figure 12).

Since it has been demonstrated that patients during febrile paroxysms following the administration of typhoid vaccine rapidly develop hypoferrremia (16) the TIBC was followed in such a patient during two paroxysms of fever (Figure 13). No significant change took place even though hypoferrremia developed.

In an attempt to determine the rapidity with which a reduction in TIBC takes place in the presence of inflammation, an abscess was produced in a dog by the intramuscular injection of staphylococci (Figure 14). Twenty-four hours after the production of the infection the SI had fallen from 130 μg per cent to 35 μg per cent. There was also a reduction in the TIBC at this time but maximum reduction did not take place until the third day at

which the SI had returned to normal. Two other dogs received repeated injections of turpentine (Figures 15 and 16). In each animal, 24 hours after an injection hypoferrremia developed and a fall in the TIBC occurred. The decrease in serum iron was always greater than the decrease in TIBC and the per cent saturation thus decreased. In each instance the maximum decrease in TIBC took place after maximum hypoferrremia had developed with the result that as the SI rose the per cent saturation increased. Following three of the six injections the serum became 100 per cent saturated during the rebound in iron content.

DISCUSSION

In 13 patients with chronic infections, in two dogs in which sterile abscesses were produced and in a single dog in which an acute infection was introduced, the total iron-binding capacity (TIBC) of the serum was reduced as compared with the normal, as was the serum iron. The reduction in the latter was proportionately greater, with the result that the per cent saturation with iron was decreased. The intravenous administration of the iron-binding protein to two patients with chronic infections increased the TIBC of the serum to nearly normal values for a period of approximately 24 hours without diminishing the rate of disappearance of iron from the serum. Furthermore, in a patient given typhoid vaccine hypoferrremia developed without a significant change in the TIBC of the serum. Again, in the dogs given injections of turpentine or staphylococci hypoferrremia preceded the maximum fall in TIBC; in both dogs and patients, even though the TIBC was diminished, the per cent saturation of the protein with iron was also reduced due to the greater fall in serum iron; and finally, there appeared to be no correlation between the degree of hypoferrremia and anemia and the degree of reduction of the TIBC. From these observations it would appear that some factor other than a reduction in the iron-binding protein is responsible for the hypoferrremia associated with infection. Since in conditions in which the demands of the body for iron are increased, namely, iron deficiency and pregnancy, there is an increase in the TIBC of the serum, and since in those disorders in which the demand of the body for iron is decreased such as pernicious anemia in relapse (12), there is a decrease in the TIBC,

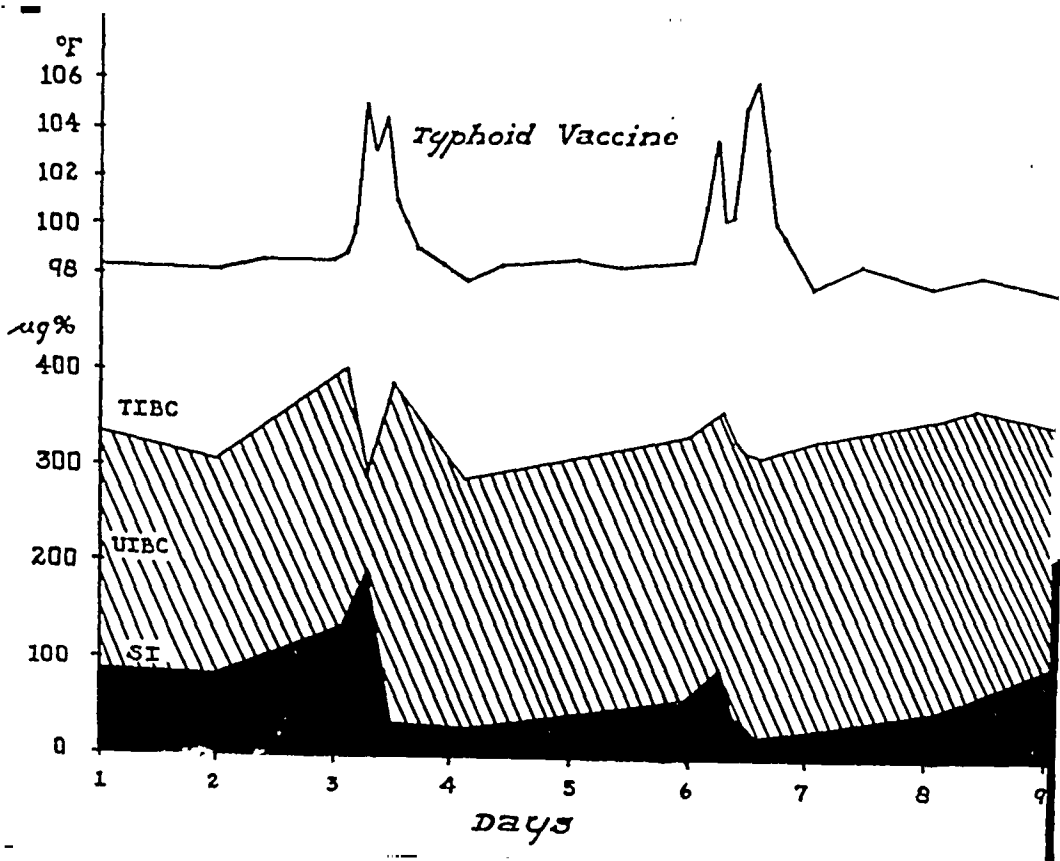


FIG. 13. THE EFFECT OF FEVER INDUCED BY THE INJECTION OF TYPHOID VACCINE ON THE TOTAL IRON-BINDING CAPACITY AND THE SERUM IRON

For symbols see Figure 5.

the question might be asked: Does the reduction in TIBC in infection reflect an attempt to reduce the intake of iron? With the information now available this question cannot be answered.

In most of the patients with infections the TIBC was reduced to about 43 per cent of the normal mean and in one patient the value was reduced 78 per cent. The total serum proteins were in general within the normal range although occasionally they were in the lower limits of normal. Thus the reduction in the iron-binding protein was out of proportion to the general reduction in serum proteins.

From our observations it would appear that after the intravenous administration of iron the height of the rise is limited by the capacity of the serum to bind iron. When the TIBC of the serum is exceeded the unbound iron rapidly leaves the blood stream and toxic symptoms develop. Since the TIBC is reduced in infections an explanation is now available for our previous observation that

the initial five-minute rise was not as great in patients with infections as in normal subjects (13).

Goetsch, Moore and Minnich (16) have observed serum iron values from 0.622 to 3.86 mg. per cent in patients following the intravenous injection of massive doses of iron (from 0.608 to 1.32 gm. as colloidal hydroxide and colloidal ferric oxide). Moore, Arrowsmith, Welch, and Minnich (17) observed serum iron values in dogs as high as 1.60 mg. per cent after the intravenous administration of 1 mg. Fe^{++} per kilogram of body weight. These experiments differ from ours in that much larger doses were used. It may be that with the administration of doses such as Moore and his associates gave, the iron combines with other proteins.

As far as our own observations are concerned, the failure of the serum iron to rise above the level of the TIBC when 0.137 mg. Fe^{++} per kilogram were given intravenously, appeared to be so consistent that we would suggest that the most

the method of determining the TIBC is by measuring the serum iron five minutes after the injection of a supersaturating amount of iron.

It may be noted in passing that, since the toxicity of injected iron seems to be dependent upon the presence of unbound iron in the serum, it is unlikely that any one particular iron compound, other than iron in combination with protein or colloidal iron, can be less toxic than another.

SUMMARY

1. The total iron-binding capacity (TIBC) of the serum was measured in 30 normal individuals. The mean \pm S.D. was $359 \pm 30.8 \mu\text{g}$ per cent. The saturation of the iron-binding protein with iron was found to be 35 ± 6.4 per cent.

2. The total iron-binding capacity of the serum was measured in 13 patients with chronic infection, in two dogs in which sterile abscesses were produced, and in a single dog given an acute in-

fection. The TIBC was found to be significantly reduced in each instance. The reduction in the serum iron was proportionately greater than the reduction in iron-binding capacity with the result that the per cent saturation was lowered.

3. The total iron-binding capacity of the serum was also measured in six patients with iron deficiency and found to be increased. The per cent saturation of the iron-binding component was less than 10 in each instance.

4. The administration of metal-combining globulin (Fraction IV-7) to two patients with chronic infections resulted in a temporary increase to normal in the total iron-binding capacity of the serum. Following the administration of this fraction the intravenous injection of iron resulted in a greater initial five-minute rise in the serum iron than previously but the rate of disappearance of the iron from the serum was not significantly affected. Furthermore, the temporary increase in

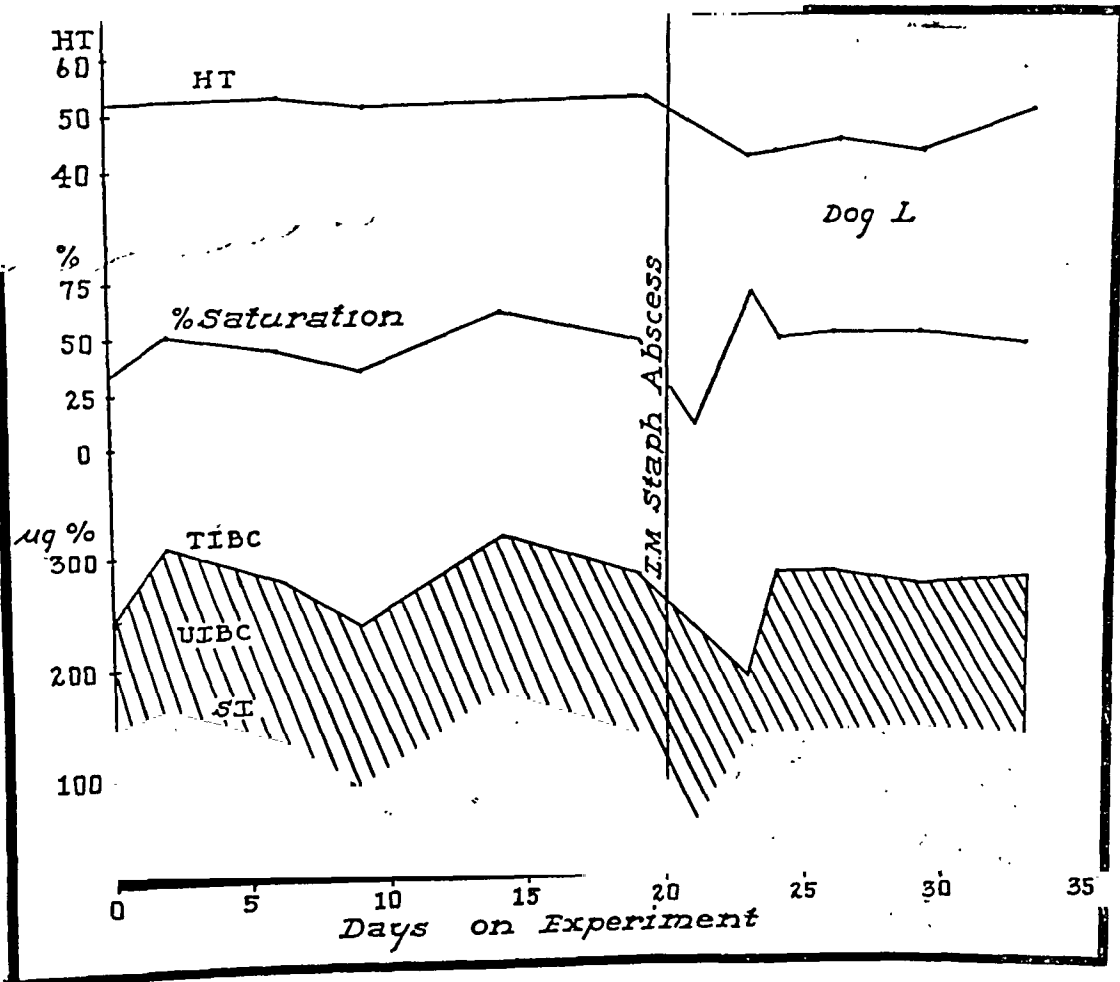


FIG. 14. SHOWING THE EFFECT OF THE INTRAMUSCULAR INJECTION OF STAPHYLOCOCCI ON THE TOTAL IRON-BINDING CAPACITY AND THE SERUM IRON IN A DOG

For symbols see Figure 5.

POTASSIUM DEFICIENCY AND THE ROLE OF THE KIDNEY IN ITS PRODUCTION^{1,2}

ANEMIA OF INFECTION—IRON-BINDING CAPACITY OF SERUM

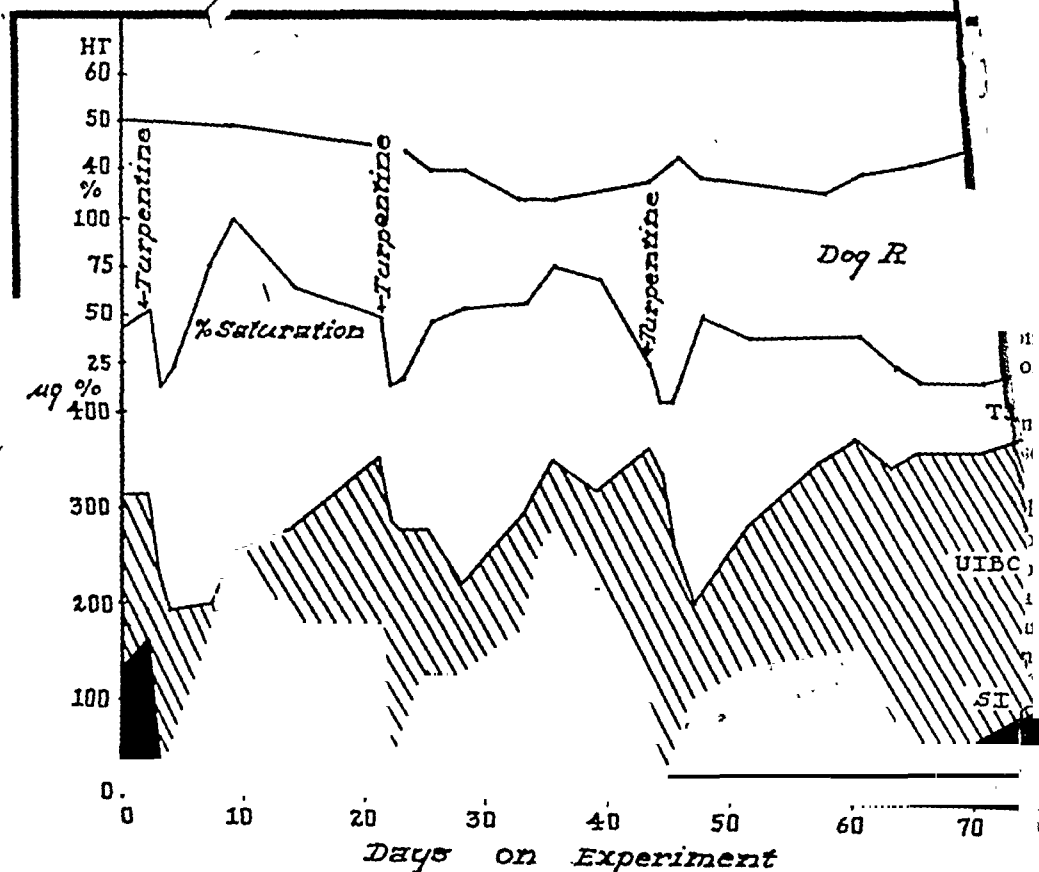


FIG. 15. THE EFFECT OF THE REPEATED INTRAMUSCULAR INJECTION OF TURPENTINE ON THE TOTAL IRON-BINDING CAPACITY AND THE SERUM IRON OF A DOG
For symbols see Figure 5.

the iron-binding capacity of the serum did not result in a detectable mobilization of iron into the blood.

5. The administration of typhoid vaccine to a patient produced a transient hypoferremia without a significant change in the iron-binding capacity of the serum.

6. These observations would indicate that the hypoferremia which accompanies infections is not the result of a reduction in the iron-binding capacity of the serum but is dependent upon some other factor.

7. The observations of Holmberg and of Laurell that the "braking" mechanism described by Waldenström is attributable to the fact that iron, in excess of that which can be bound, rapidly leaves the blood stream, and their conclusion that the intravenous administration of iron in excess of the amount which can be bound produces toxic symptoms, have been confirmed.

ACKNOWLEDGMENT

For the supply of Fraction IV-7 as well as cryo-metal-combining globulin, we are indebted to J. Cohn, Harvard Medical School, Boston, Massachusetts.

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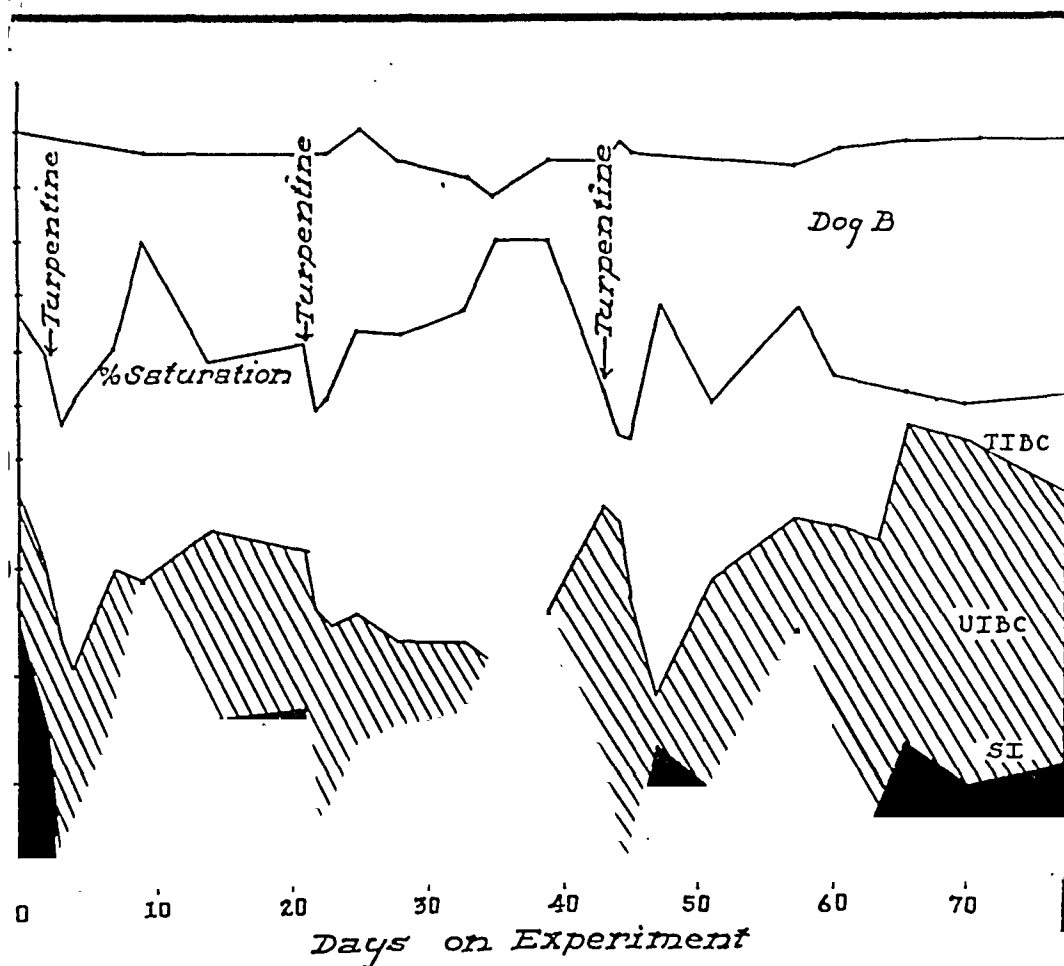


FIG. 16. THE EFFECT OF THE REPEATED INTRAMUSCULAR INJECTION OF TURPENTINE ON THE TOTAL IRON-BINDING CAPACITY AND THE SERUM IRON OF A DOG

For symbols see Figure 5.

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POTASSIUM DEFICIENCY AND THE ROLE OF THE KIDNEY IN ITS PRODUCTION^{1,2}

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It has been recognized that a depression of the concentration of potassium in serum may reflect a concomitant deficiency of potassium in the cells of the body (1). Low concentrations of potassium in serum and diminished potassium contents of skeletal muscle have been reported in experimental animals maintained on diets deficient in potassium (2) and in animals treated with large doses of desoxycorticosterone (3). Depressions of the concentration of potassium in serum have been observed clinically in patients receiving desoxycorticosterone (4), in familial periodic paralysis (5, 6), in diabetic acidosis (7, 8), in intestinal obstruction (9), and in diarrhea (10). In some of these conditions deficit of cellular potassium has been indicated by negative balances of potassium when the intake of potassium was low and retention of potassium when the ion was administered (10-13). However, significant alterations in the cellular content of potassium may occur without much change in the amount and concentration of potassium in the extracellular fluid and serum (10, 14). Levels of serum potassium therefore, may, but do not necessarily, reflect the state of depletion or repletion of cellular potassium.

The study reported here was undertaken in an attempt to elucidate some of the clinical problems of potassium deficiency in adult patients. These problems include the relation of the concentration of potassium in serum to cellular deficiency of the ion, the extent of such cellular deficiencies and their relation to retention of administered potassium, and the physiological abnormalities by which such deficits are produced.

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² Read in abstract form before the annual meeting of the American Society for Clinical Investigation, Atlantic City, May 3, 1948.

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EXPERIMENTAL MATERIAL AND PROCEDURE

Six adult patients on the medical and surgical services of the New Haven Hospital were studied. Of these, five were suffering from conditions involving extensive losses of fluids from the gastrointestinal tract, and the sixth had a cerebral vascular disturbance with quadriplegia. All the patients were being sustained wholly or in part by parenteral fluids with minimal amounts of potassium. Four of the patients with gastrointestinal fluid loss had abnormally low concentrations of potassium in serum.

In all patients exchanges of chloride, sodium, potassium and nitrogen were measured during a series of periods of 24 hours or longer. In five of the patients these measurements were made in one or more preliminary periods during which a negligible amount of potassium was given. These were followed by periods of potassium administration. In Case 1, A. M., potassium was given in the initial period. In five cases the potassium was given parenterally as K_2HPO_4 and KH_2PO_4 in molar ratio of 3.55 to 1 to yield a pH of 7.35; in the sixth case it was given as KCl. These potassium salts, added to the other intravenous fluids in concentrations up to 70 m.eq. per liter, were given slowly at a rate not in excess of 20 m.eq. per hour. The amount of potassium given by the intravenous route varied from 29 to 167 m.eq. per day. Water and sodium chloride were administered according to the clinician's opinion of the various requirements of the moment; before potassium therapy was instituted, two of the patients were maintained on intakes high in sodium (J. L., E. R.), and four on intakes low in sodium in varying degrees. All of the patients were given carbohydrate and casein hydrolysate during most of the periods. Clinical diagnoses are appended to Table I.

Two normal control subjects were maintained for three successive 24-hour periods on a liquid diet of milk fortified with "Dextri-Maltose." This diet was isocaloric with the usual daily intake of the subjects and kept them approximately in nitrogen equilibrium. On the second of the three days, 10 grams of KCl were added to the diet to ascertain whether retention of potassium could be induced under these circumstances in a normal subject during a 24-hour period or longer.

One normal control subject was also given an isocaloric diet low in potassium for two 24-hour periods. The diet consisted of a solution of casein, lactose, butter and sodium chloride. The experiment was designed to determine the minimal rate of renal excretion of potas-

TABLE I
External exchanges of water, electrolytes, and nitrogen

Subject	Period	K* given	Intake						Output						Balance†			
			Urine						G.I. fluid†									
			H ₂ O	Cl	Na	K	N	CH ₂ O	Vol.	Cl	Na	K	N	Vol.	Cl	Na	K	N
	dates	grams	cc.	m. eq.	m. eq.	m. eq.	grams	grams	cc.	m. eq.	m. eq.	m. eq.	grams	cc.	m. eq.	m. eq.	m. eq.	grams
A. M. Case 1	11/3-4	6.4	3950	96	127	165	6.2	375	1290	50	22	29	7.6	1960	227	236	14	3.3
	11/4-5	3.2	5050	178	250	87	15.2	350	1190	4	31	46	9.9	2450	260	310	15	3.0
	11/5-6	6.4	6350	346	408	167	12.4	425	1380	5	58	68	11.7	2650	272	326	15	2.8
	11/6-7	3.2	6610	410	482	87	15.2	425	1580	8	68	61	12.5	2960	295	361	16	2.9
	11/7-8	3.2	8050	504	593	86	20.5	500	1240	5	43	52	11.5	3720	391	478	19	2.9
	11/8-9	1.6	7950	504	593	45	20.5	450	3700	54	111	47	14.3	3200	342	403	12	3.1
	11/9-10	3.9	5350	358	419	103	14.3	295	1300	20	49	46	7.6	3000	332	369	15	2.3
E. R. Case 2	11/17-18	2.9	7050	573	612	79	26.6	395	940	87	56	24	12.3	4700	421	594	41	4.8
	11/18-19	3.0	7150	576	617	83	26.0	375	1680	204	126	44	15.2	3810	382	475	28	4.6
	11/19-20		7000	545	586	6	25.8	400	2330	240	172	36	17.4	1140	123	138	7	0.9
	11/20-21		5100	395	438	5	22.6	275	2800	336	277	28	15.7	280	29	37	1	0.3
	11/21-22	3.0	4800	323	339	80	20.4	320	2720	307	254	27	12.5	870	26	80	10	4.2
	12/22-23		3690	0	222	0	0	150	1750	132	65	50	15.1	125	7	9	2	0.5
	12/23-24		3025	231	231	0	0	150	3420	356	250	59	13.5	870	82	104	6	0.8
J. L. Case 3	12/24-25	1.1	3200	212	243	29	6.2	175	2500	221	197	35	8.4	0				
	12/25-26	1.1	4130	192	254	31	12.4	350	3380	251	290	33	14.6	0				
	12/26-27	3.2	4120	194	255	88	12.7	300	2030	109	130	15	5.8	0				
	12/27-28	3.2	3160††			147	8.7		2415	97	194	35	7.7	0				
R. W. Case 4	1/28-29		4120	269	469	4	12.4	100	1750	8	64	85	24.0	590	61	59	9	1.8
	1/29-30		3160	192	432	4	12.4	100	900	8	54	45	15.4	350	45	49	2	0.4
	1/30-31	6.4	3990	96	216	166	6.2	212	1500	17	121	60	18.9					
	1/31-2/1	3.4	1835	30	61	91	6.6	63	700	9	136	31	9.0					
	2/2-4		5220	134	227	6	18.6	240	2300	134	195	20	16.3	200**	25	26	0	0
	2/4-6		5100	82	144	11	11.8	200	2770	70	111	11	10.6	312	39	40	1	0
	2/6-7	4.2	2400	141	65	109	6.2	90	830	62	78	10	3.2	340	25	27	2	0.1
	2/7-8		2250	103	134	2	6.2	100	2370	46	158	28	8.9	250	19	19	1	0.1
	2/8-9	4.2	2950	228	162	110	9.0	100	2510	232	224	69	7.1	40	1	1	0	0
	2/10-12		4500	269	331	4	12.4	200	2985	196	231	28	13.0	375	44	42	4	0.5
	2/12-13	4.2	2200	126	50	109	6.2	110	1015	114	114	29	10.5	120	5	13	11	0.1
	2/13-14	2.6	2100	86	50	69	6.2	75	2175	199	197	43	9.9	0	-115	-150	26	3.7

Patients

TABLE I—Continued

Subject	Period	K* given grams	Intake							Output							Balance†				
			Urine							G.I. fluid†											
			H ₂ O	Cl	Na	m. eq.	K	N	CHO	Vol.	Cl	Na	m. eq.	K	N	grams	Vol.	Cl	Na	m. eq.	K
M. McK. Case 5	dates 12/3-4 12/4-5	grams 3765 3760 6.4	m. eq. 23 23	m. eq. 81 81	m. eq. 3 168	grams 14.3 14.3	grams 218 230	cc. 2310 1375	m. eq. 2 3	m. eq. 6 5	m. eq. 27 19	grams 12.7 6.3	cc. 300 540	m. eq. 4 2	m. eq. 8 5	grams 1.1 2.9	m. eq. + 15 + 15	m. eq. + 64 + 67	m. eq. - 25 + 146	grams + 0.5 + 5.1	
	1/15-16 1/16-17 1/17-19		77 77 153	139 139 277	4 4 8	12.4 12.4 24.8	150 150 300	1670 1400 2900	51 37 66	110 81 176	43 47 79	25.2 21.9 47.6	0 0 0				24 + 38 + 84	26 + 55 + 97	39 - 43 - 71	- 12.8 - 9.5 - 22.8	
	C. L. Case 6	1/22-23 1/23/24	2.1 4.2	77 77	139 139	58 113	12.4 12.4	150 150	1410 1910	86 144	168 242	43 91	20.0 23.7	0 0				- 12 - 72	33 - 110	15 + 22	- 7.6 - 11.3
R. E.	12/16-17 12/17-18 12/18-19	5.2	112 246 112	86 86 86	116 250 116	15.1 15.1 15.1	434 434 434	2270 1710 1700	159 235 108	124 111 53	116 218 118	13.4 13.2 13.3	100†† 105 50	1 5 0	5 10	1.3 0.4	8 + 2 + 2	33 + 21 + 7	0 21 7	+ 0.6 + 1.4	
	12/16-17 12/17-18 12/18-19	5.2	112 246 96	86 86 74	116 250 99	15.1 15.1 13.0	434 434 372	1210 1190 1100	161 209 87	123 72 38	103 214 113	15.5 13.3 12.4	100 500 300	0 8 8	13 10	2.9 1.7	51 + 35 - 1	39 + 1 + 24	13 + 12 - 30	0.4 - 1.1 - 1.1	
	R. E.	1/28-29 1/29-30		90 74	71 84	2 2	12.8 13.5	350 400	2280 1860	125 77	114 80	55 34	12.1 12.7	300	0	13	1.1	37 - 5	45 - 11	53 - 49	+ 0.7 - 0.3

* Subjects R. W., R. E., and R. T. were given KCl. The others received K as buffered KH_2PO_4 and K_2HPO_4 .

† Includes fluids lost by rectum as well as from upper gastrointestinal tract.

‡ Balances of Cl and Na corrected for small quantities lost in serum drawn for analysis.

§ Intake includes small quantities of food, the composition of which was estimated from Sherman's tables (17).

** Composition of this sample of gastric juice is assumed from subsequent analyses since specimen was discarded.

†† Composition of food estimated from Sherman's tables (17).

‡‡ Not analyzed.

Balance data are expressed per individual period rather than cumulatively.

Diagnoses:

A. M., Case 1, ♂, 63 yrs. Carcinoma of biliary tract; post-operative, external biliary and small intestinal fistula.

E. R., Case 2, ♀, 25 yrs. Vomiting of unknown origin.

I. L., Case 3, ♂, 64 yrs. Adenocarcinoma of the colon; intestinal obstruction.

R. W., Case 4, ♀, 26 yrs. Partial duodenal obstruction; mongolian idiocy.

M. McK., Case 5, ♀, 60 yrs. Cirrhosis of liver; vomiting and diarrhea.

C. L., Case 6, ♂, 58 yrs. Cerebrovascular accident with quadriplegia.

TABLE II
Analyses of blood and serum, and changes in the distribution of sodium and potassium between the phases of body fluid

Subject	Time	K given	Weight	Blood NPN	Serum						Extra-cellular volume	Change in				
					CO ₂	Cl	Na	K	H ₂ O*	Total protein		Cellular Na	Extra-cellular K	Total	With N ₂	In excess of N
	date	m. eq.	kilo.	mg./100 cc.	m. eq./liter	m. eq./liter	m. eq./liter	m. eq./liter	grams/liter	grams/100 cc.	liters	m. eq.	m. eq.	m. eq.	m. eq.	m. eq./kilofl.
A. M. Case 1	11/3			41	36.1	90.0	141.5	2.0	943	5.64	(9.0)	+40	+7	+115	+7	
	11/4	163		36	38.6	89.4	143.2	3.2			7.1	+5	+4	+30	+2	
	11/5	82		38	37.0	86.2	141.4	3.0	936	6.52	7.7	+68	+7	+77	+5	
	11/6	163		38	35.7	86.5	140.7	3.7			8.8	+87	+7	+3	+0	
	11/7	82		37	29.2	84.2	138.5	4.0			10.1	+34	+1	+16	+18	
	11/8	82		33	29.1	95.0	130.5	3.4	937	6.44	9.9	+36	+9	+10	+7	
	11/9	41	51.0		28.1	91.8	137.0	3.0			11.5	+137	+16	+26	+10	
	11/10	100					139.5	3.6								+4.6
	11/17		49.0	41	22.0	99.5	139.2	3.5	937		(11.5)	+47	+8	+6	+23	
	11/18	74			21.1	102.3	137.3	4.1			11.7	+35	+1	+12	+13	
E. R. Case 2	11/19	77			22.7	99.3	136.8	3.9		6.45	12.0	+40	+1	+38	+18	
	11/20			41	23.7	99.4	136.9	3.5			13.6	+3	+1	+25	+16	
	11/21		51.5	41	25.1	94.9	136.4	3.3			14.7	+3	+0	+43	+15	
	11/22	77		34	25.2	94.6	134.4	3.3			14.7	+3	+0			
	12/22			85	9.9	107.8	128.9	6.6	952	4.38	10.5	+26	+37	+15	+4	
	12/23			35	22.1	88.9	135.0	2.8			11.3	+160	+13	+52	+31	
	12/24			28	30.8	95.2	144.0	2.2			8.6	+150	+3	+9	+5	
	12/25	27				101.0	141.5	2.8	950		(8.0)	+64	+3	+1	+6	
	12/26	27	40.2	29	30.5	93.7	144.5	2.4		5.40	8.7	+28	+3	+70	+21	
	12/27	82	40.4	23	29.6	95.2	143.7	2.5	945		8.3	+27	+6	+106	+2	
J. L. Case 3	12/28	82		21	26.4	99.9	135.8	3.4			8.3	+27	+6	+181	+54	
	12/30	2408				100.7	137.3	3.4								
	1/28			40	30.2	98.0	136.2	3.6			6.1	+32	+4	+86	+31	
	1/29			39	27.6	99.8	148.0	2.4	944	5.56	7.7	+167	+3	+46	+9	
R. W. Case 4	1/30			40	31.2	107.3	156.4	2.5			8.3	+4	+8	+98	+22	
	1/31	164	45.4	29	28.3	105.6	152.2	3.2			(9.1)		+12	+48	+6	
	2/1	86				105.6		4.4			9.3					+3.8
	2/2		45.5	25	18.4	101.4	143.0	3.3	939		(9.0)	+48	+3	+17	+4	
	2/4		45.0	27	27.3	100.5	140.7	3.7	939		8.8	+77	+1	+2	+6	
	2/6		44.5	23	28.9	99.4	134.0	4.0	932		8.5	+161	+4	+93	+8	
	2/7	107	44.0	22	26.5	95.6	133.8	4.0	930		9.4	+174	+4	+28	+8	
	2/8		43.1	25	—	97.1	140.0	4.1	920		9.4	+121	+1	+39	+19	
	2/9	107	43.1	24	26.1	100.9	139.0	4.5	929		9.1	+1	+2	+39	+5	+2.3

Patients

TABLE II—Continued

	Subject	Time	K given	Weight	Blood NPN	Serum						Extra-cellular volume	Change in					
						CO ₂	Cl	Na	K	H ₂ O*	Total protein		Cellular Na	Extra-cellular K	Cellular K			
															Total	With N ₂	m. eq.	m. eq.
Patients	R. W Case 4 Contl.	2/10		25	24.2	m. eq./ liter 95.6	m. eq./ liter 135.7	m. eq./ liter 3.8	grams/ liter 923	grams/ 100 cc. 9.1	m. eq. - 8	m. eq. - 36	m. eq. - 3	m. eq. - 33	m. eq.	m. eq.	m. eq.	m. eq.
		2/12		25	—	96.5	141.5	4.4	925	9.7	+ 3	+ 66	- 13	+ 79	↓	↓	↓	↓
		2/13		29	23.9	97.9	140.6	4.7	928	9.7	- 6	+ 32	- 8	+ 40	+ 119	+ 40	+ 119	+ 2.8
		2/14		28	25.7	97.7	137.9	4.6	928	8.6								
	M. McK. Case 5	12/3		33	30.5	82.8	132.5	3.6	939	(16.0)	- 4	- 21	+ 2	- 23		- 23		+ 2.1
		12/4		32	32.3	82.5	134.4	3.3	935	16.2	+ 14	+ 132	+ 14	+ 118		+ 118		
	C. L. Case 6	12/5		29	32.4	80.3	130.5	4.0		6.70								
		1/15		55	27.2	102.8	145.0	4.8	937	(15.0)	0	- 39	- 30	- 9		- 9		
		1/16		51	101.8	140.8	140.8	4.7	937	15.3	- 12	- 31	- 18	- 13		- 13		
		1/17		50	109.2	146.8	146.8	4.1	928	14.7	+ 19	- 90	- 53	- 37		- 37		
Controls	R. E.	1/19			105.8	151.7	151.7	5.0		15.7								
		1/22		44	25.6	104.0	147.3	4.1	931	(15.0)	7	+ 8	- 28	+ 36	↓	+ 36	↓	+ 1.2
		1/23		53	25.8	106.4	144.7	4.7	932	14.6	+ 4	+ 18	- 19	+ 37	+ 73	+ 37	+ 73	
		1/24		46	24.8	102.3	142.7	5.1	932	14.6								
	R. E.	12/16		36		101.3	140.3	4.1	941	(14.8)	0	0	+ 6	- 6		- 6	↓	↓
		12/17		73.2	34	101.5	136.2	4.3	932	14.2	+ 2	+ 19	- 2	+ 21	+ 8	+ 21	+ 8	+ 0.1
		12/18		73.0	37	99.0	143.3	4.3	939	14.7	- 4	- 3	+ 5	- 8	+ 10	+ 15	+ 10	
		12/19		73.0	36	100.7	142.3	4.1	933	14.4								
	R. T.	12/16		38		101.7	140.5	4.5	931	(11.4)	- 2	+ 15	+ 5	+ 10	+ 15	+ 10	↓	↓
		12/17		57.0	32	97.7	139.3	4.4	939	11.2	+ 2	+ 10	+ 5	+ 15	+ 19	+ 15	+ 19	+ 0.1
12/18			57.2	35	98.5	136.1	4.5	935	11.4	- 5	- 22	- 3	- 19	- 19	- 19	- 19		
R. E.	12/19		56.6	36	96.8	139.7	3.8	933	11.7									
	1/28		74.1	31	96.6	139.7	4.3	933**	(14.8)	- 6	- 47	+ 5	- 52	- 47	- 52	- 47	- 52	
	1/29		73.0	28	91.8	142.2	3.8	933**	15.2	- 5	- 44	- 5	- 39	- 44	- 39	- 44	- 39	
	1/30		72.2	31	95.8	142.5	3.6	933**	14.6	+ 69								

* Determined directly or calculated from the concentration of serum protein.

† Calculated by changes in chloride balance forward and backward from an assumed extracellular volume (designated by parentheses).

‡ The balance of protein is corrected for change in NPN throughout an assumed volume of body water.

§ Estimated from Sherman's tables (17).

** Assumed.

†† Cumulative changes during potassium administration. Except where indicated balance data are expressed per individual period rather than cumulatively. Time as designated by date indicates end of period at which time serum analyses were made and balances for the period determined.

sium in a subject in nitrogen equilibrium and not initially depleted of potassium.

METHODS

The amounts of potassium and sodium in serum, urine, and other body fluids were measured with the flame photometer (15). The other chemical methods which were used, and the methods of calculation of the derived data, have been previously reported (14, 16). In the present study the concentration of potassium in extracellular fluid was assumed to be the same as that in serum because of the uncertainty concerning the proportion of the latter which is non-diffusible. Any error in this assumption would have little effect on the calculated transfers of intracellular potassium. In order to differentiate at least to some extent between transfers of potassium which are associated with cellular anabolism or catabolism and those which are not, a relationship of potassium to nitrogen

must be assumed. In the former studies just cited a ratio was used of potassium to nitrogen of 2.4 m.eq. to 1 gram; the same ratio has been employed in the present calculations to determine the movements of potassium in "excess" of nitrogen. Use of the K:N ratio of 3:1 as advocated by Darrow (10) and which probably represents the upper limit of this ratio, would increase the magnitude of the change in cell potassium associated with nitrogen by 20 per cent, but would not significantly alter the direction of change of excess cell potassium or the conclusions based thereon.

RESULTS

The analytical and derived data are presented in Tables I and II and in Figures 1 to 6.

The patients retained a significant portion of administered potassium in the cellular phase in ex-

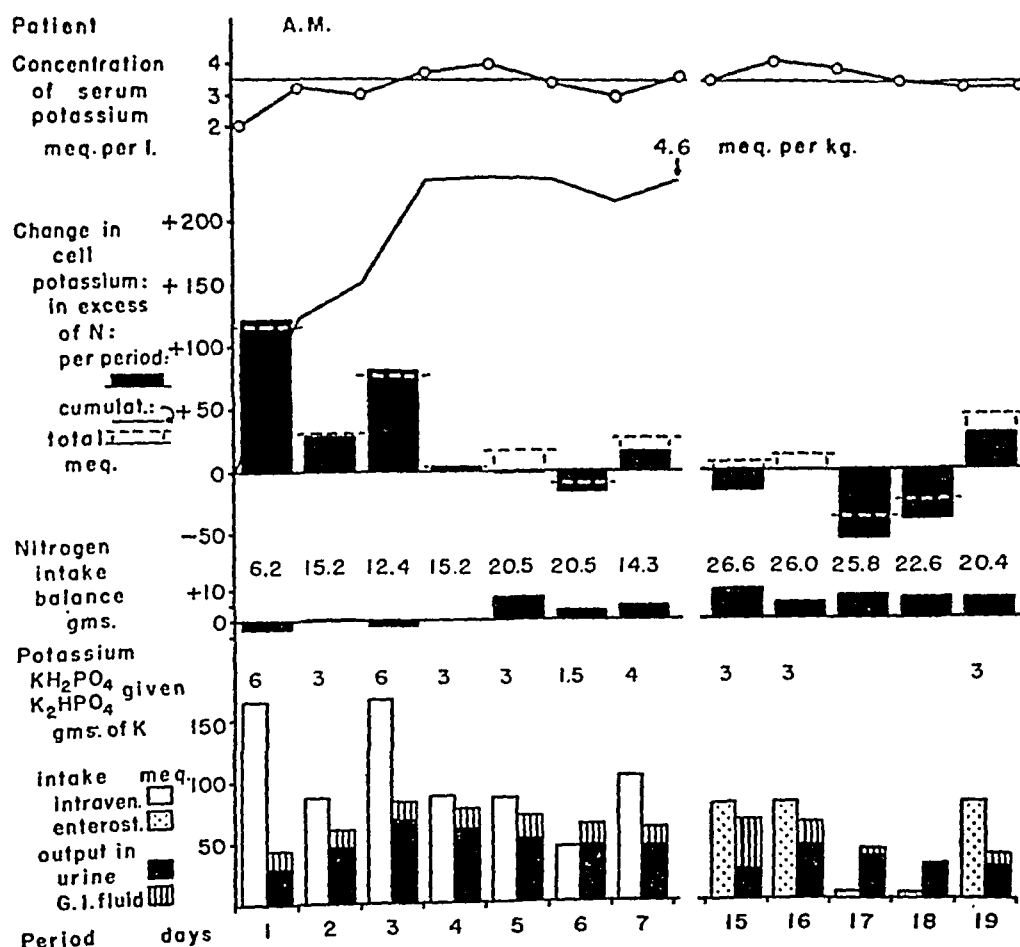


FIG. 1. EXCHANGES OF POTASSIUM AND NITROGEN IN A PATIENT TREATED WITH POTASSIUM PHOSPHATE

Two periods are shown of potassium administration to a patient (A. M., Case 1) with a duodeno-biliary external fistula who received nothing by mouth for 34 days. On Days 1 to 7 inclusive, potassium phosphate was given by vein, on Days 15, 16, and 19 by enterostomy.

Potassium retention in the cellular phase was maximal during the first three days; little was retained thereafter. When potassium was stopped during Days 17 and 18 urinary excretion of potassium persisted. Except for one 24-hour period, more potassium was invariably excreted in urine than in gastrointestinal fluid.

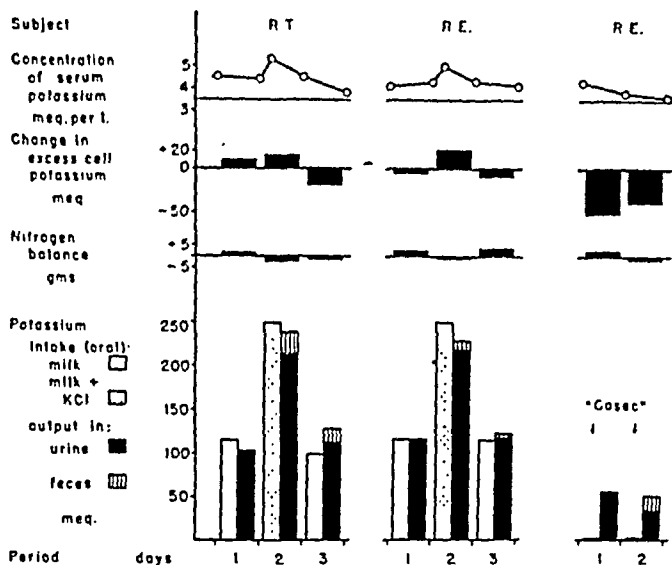


FIG. 2. EXCHANGES OF POTASSIUM IN TWO CONTROL NORMAL SUBJECTS ON HIGH AND LOW INTAKES OF THE ION

In all three experiments the subjects were on isocaloric diets. In the first two experiments the moderate oral intake of potassium in milk was supplemented on Day 2 with KCl. No significant amounts of potassium were retained. In the third experiment, despite the low intake, potassium continued to be excreted in the urine and a cellular deficit was initiated.

ess of nitrogen. As the control subjects did not, this retention presumably indicates a pre-existing cellular deficit of the ion. The magnitude of the deficit was not defined by the extent of retention in each patient, however, since only in Case 1, A. M., was potassium administered for a sufficient length of time to demonstrate that the patient's cells were approaching saturation (Figure 1); the other patients were still retaining potassium at the end of the study. The amount of potassium so retained ranged between 73 and 232 m.eq., or 1.2 to 4.6 m.eq. per kilogram of body weight (Table II). The upper limit of this range would be increased to 283 m.eq., or 7.0 m.eq. per kilogram, if the last two days of Case 2, E. R., are included when the intake of potassium in food was calculated from Sherman's tables (17). The two normal subjects who were given somewhat larger quantities of potassium, retained 0.1 m. eq. per kilogram each over the three 24-hour periods; essentially all of the potassium given was excreted (Table II, Figure 2).

The cellular deficits of potassium as demonstrated by retention of the ion were usually, but not always, associated with a low concentration of

serum potassium; when the concentration of potassium in the serum was low a cellular deficit was always present (Figure 3, [a]). When the cellular content of potassium was increased, both the serum concentration and the absolute amount of extracellular potassium tended to rise; but considerable alteration occurred in the former fraction of potassium with relatively minor changes in the latter two values (Figure 3, [b and c]).

The major factor in the development of deficits of potassium in these patients appeared to be the continued excretion of potassium by the kidney during times of restricted ingestion of the ion. Since these patients were not studied quantitatively from the time of onset of their disease, the data do not contain complete evidence concerning the mode of origin of the potassium deficits. Loss of potassium by vomiting and diarrhea must have been a contributory factor. In Cases 1 to 5 fluid had been lost steadily from stomach or upper small intestine for periods of four days to three months, immediately preceding the studies. Direct analyses of samples of such fluids in four of these cases revealed potassium concentrations one to six times as high as those of normal serum, and losses of as

much as 41 m.eq. per day (Case 1, A. M.). As the potassium intake of vomiting patients is low, a negative balance may ensue and reach significant proportions over a period of days.

However, during the periods studied, with one exception, more potassium was lost in urine than in gastrointestinal fluid (Table I). In both the patients and the normal subjects the capacity of the kidneys to conserve the ion was limited. In Figure 4 the line which defines the relation between the rate of administration and the rate of excretion of potassium in the normal subjects, does not pass through the origin but intersects the abscissa at approximately 40 m.eq. per day. Some of the patients on low intakes excreted less potassium, but the ion never disappeared from the urine. In three of these patients (A. M., R. W., M. McK.)

who were in nitrogen equilibrium the conditions were optimal for renal conservation of potassium. Yet the rates of potassium excretion in these patients were 28, 27, and 5.5 m.eq. per day, and the lowest concentration ratio of urine to serum (U/P) was 1.0. Under these conditions and in these patients, there was no direct evidence of active tubular reabsorption of potassium against a concentration gradient.

The height of the concentration of potassium in serum was less well correlated with the rate of excretion in the patients than in the normal subjects (Figure 5, Tables I and II). In some of the former, more potassium appeared in the urine when the serum concentration was low than when it was at higher levels; in the latter, slight increases in the serum levels were associated with

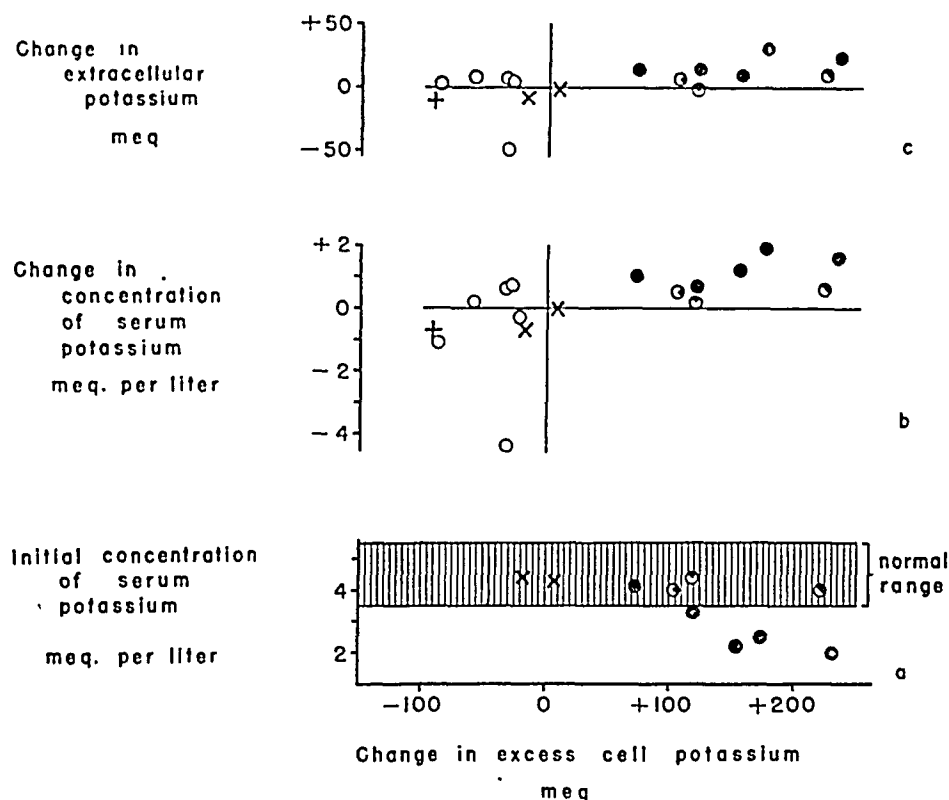


FIG. 3. COMPARISON OF CHANGES IN EXCESS CELL POTASSIUM WITH (A) THE INITIAL LEVEL AND (B) THE CHANGES IN CONCENTRATION OF SERUM POTASSIUM, AND (C) WITH THE CHANGES IN AMOUNT OF EXTRACELLULAR POTASSIUM

The control subjects are represented by crosses, the patients by circles. Open circles represent the total changes for consecutive periods when the intake of potassium was low, closed or partially closed circles represent similar periods when the intake was high. The half-solid circle is the probable summation of two periods of high potassium intake (quarter-solid circles) in R.W., Case 4.

Retention of cellular potassium is roughly correlated with an initial low level of serum potassium (a), with a rise in serum potassium concentration (b), and with small increases in the amount of extracellular potassium (c).

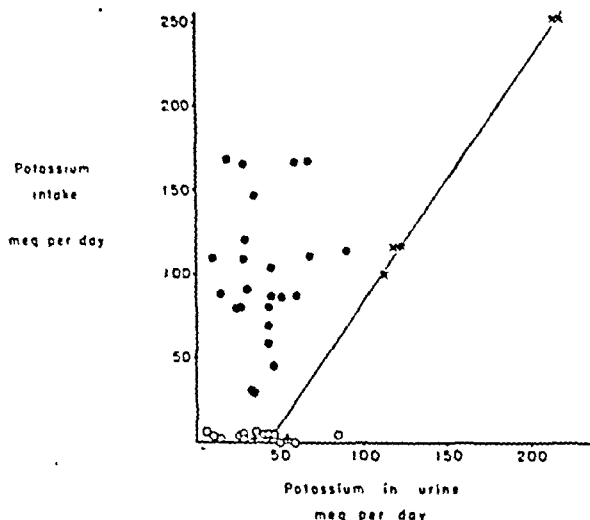


FIG. 4. RELATION OF THE DAILY EXCRETION OF POTASSIUM IN URINE TO THE DAILY INTAKE OF POTASSIUM

Data are for 24-hour periods, crosses represent periods of low (+) and high (x) intake of potassium in the normal subjects. Open and solid circles represent similar periods, respectively, in the patients.

Potassium excretion varied directly with the intake of the ion in the normal subjects but not in the patients. In both groups, the excretion of potassium persisted when the intake was negligible.

large increases in the renal excretion of the ion. The concentration of serum potassium was measured at intervals of 24 hours; levels at intermediate points closer to the actual time of potassium administration were probably higher, at least in the normal subjects. Failure of the serum concentra-

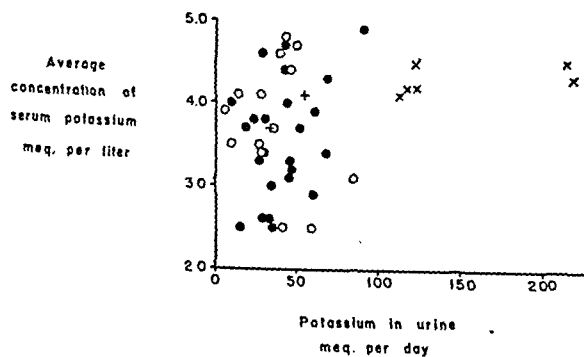


FIG. 5. RELATION OF THE DAILY EXCRETION OF POTASSIUM IN URINE TO THE AVERAGE CONCENTRATION OF POTASSIUM IN SERUM

Symbols are the same as in Figure 4. In the normal subjects, slight increases in serum concentration were associated with large increases in the rate of excretion of potassium. This relationship did not hold in the patients.

tion to rise to comparable intermediate levels might explain the failure of the patients to excrete as much potassium as did the controls. In the former the amount of potassium excreted in the urine bore no relation to the amount of potassium given (Figure 4). In four of the patients actually less potassium was excreted during periods of high intake than during those of low intake. Unfortunately our data cannot define completely the role of the serum potassium level in this phenomenon, but presumably the primary cause for the failure of the patients to excrete administered potassium was the avidity of the cells of other tissues for the ion.

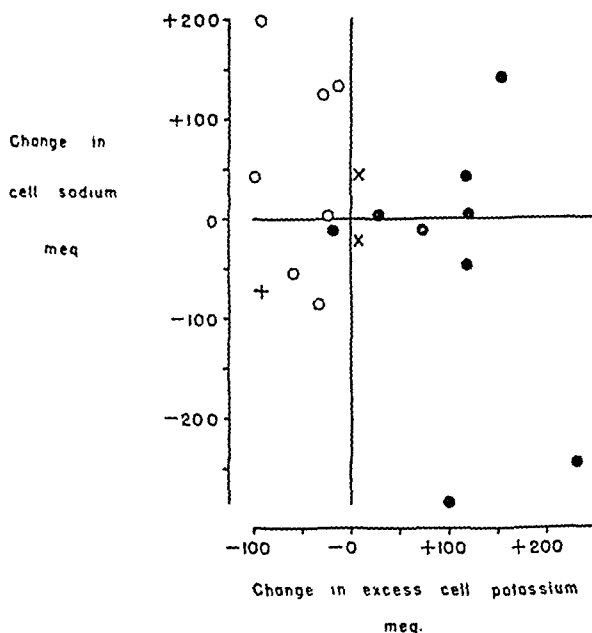


FIG. 6. COMPARISON OF CHANGES IN EXCESS CELL POTASSIUM AND IN CELL SODIUM

Symbols are the same as in Figure 3, except that the summation of periods in Case 4, R.W., is omitted. An inverse relationship is present in only a few of the periods.

Disturbances in the distribution of other electrolytes could not be causally related to the potassium deficiencies in our patients. There was no consistent relationship between the concentrations in the serum of sodium or chloride and those of potassium (Table II). An inverse relationship between changes in cell sodium and changes in cell potassium was present in only a few of the patients (Figure 6). The CO_2 content of serum was abnormally high at some stage in the course

of these studies in four of the six patients, but only in Case 1 was it clearly unrelated to the intravenous infusion of alkali or to a possible primary CO_2 excess (Table II). The administration and retention of potassium did not appear to alter significantly the nitrogen balance. Only in two cases (Cases 3 and 5) did the latter become less negative or more positive when potassium was given while the nitrogen intake was maintained at the same level (Table I).

Electrocardiographic and clinical findings in these patients were surprisingly non-specific with respect to potassium deficiency. In the patients with low concentrations of serum potassium, electrocardiographic abnormalities, *e.g.*, depression of the T waves, were noted which were similar to those previously reported (18, 19). There was no precise correlation between the level of serum potassium and the degree of electrocardiographic disturbance. During the course of infusions of potassium these abnormalities sometimes receded but no cardiotoxic effects supervened. No definite symptoms or physical signs of neuromuscular disturbance, respiratory embarrassment, or specific cardiac abnormality were observed, despite the fact that four of the patients had concentrations of serum potassium in the range of those reported in familial periodic paralysis (5) and in other patients in whom paralytic phenomena were attributed to hypokalemia (7, 20, 21). No detrimental effects of potassium administration were seen with the following exceptions: in Case 1 more fluid was lost through the rectum when potassium phosphate was added to the fluid given through the enterostomy, and diarrhea occurred in one of the normal subjects following the ingestion of potassium chloride. Otherwise, the patients tolerated the potassium therapy well.

DISCUSSION

Limits and sources of error. The magnitude of changes observed in these studies greatly exceeded any possible sources of errors in chemical determinations. This was also true with respect to the collection of specimens despite the fact that the patients were studied on the open ward. Electrolyte losses in sweat were not measured. As the patients were not sweating profusely, this neglect as far as potassium is concerned would be of minor importance (22); in the case of chloride and so-

dium, the error might be greater, but probably would not significantly alter results.

The derived data were calculated on the basis of certain assumptions which are open to question. Changes in extracellular potassium were estimated from alterations in the chloride space. Admittedly some chloride is intracellular but its magnitude is small. In addition the extracellular potassium is a relatively minor fraction of total body potassium. The questionable validity of equating the chloride space with the extracellular fluid, therefore, does not invalidate the derived data on exchanges of cellular potassium. But the relatively large extracellular component of sodium does mean that the estimated changes in cellular sodium are only rough approximations.

A relationship of potassium to nitrogen has been assumed in order to calculate which transfers of cellular potassium were due to tissue anabolism or catabolism, and which were occurring in intact tissue cells. Such K:N ratios as have been used for this purpose have been derived either from tissue (skeletal muscle) analyses or from the excretion of these substances by fasting subjects (16, 23). It is possible that this relationship may vary widely in other circumstances (24, 25, 26). But the K:N ratio was used as the only available yardstick to permit at least some differentiation between these two types of transfers of cellular potassium. In any case the uncorrected change in cellular potassium was in the same direction as significant changes in excess potassium in all the subjects studied, and the correction for potassium associated with nitrogen was small in most instances.

Relation of retention of potassium to deficit of the ion. The two control studies indicate that extra potassium given to a normal subject is not retained. Similar evidence has been found in previous investigations for both shorter and longer periods of time (27 to 30). Since the potassium was administered to both the patients and the control subjects in comparable amounts, and over a comparable period of time, there appears to be some justification in equating retention of the ion with a pre-existing deficit.

The patients retained 1.2 to 7.0 m.eq. of excess cell potassium per kilogram. Darrow's infants with diarrhea retained 5.8 to 17.3 m.eq. per kilogram (10). However, the estimation of a deficit by the retention of an administered substance re-

quires saturation of the subject; *i.e.*, it must be given until no more is taken up by the cells. Since only one patient, Case 1, (A. M.), was so saturated, the positive balances of potassium exhibited by the other patients probably did not measure the full extent of their potassium deficits.

Mechanism of potassium depletion. During the periods of study our patients lost smaller quantities of potassium in gastrointestinal fluids than in urine, although larger quantities may have been lost via the gastrointestinal tract during the periods preceding the studies. Undoubtedly this latter route of loss may be of greater importance in many cases of diarrhea (10), steatorrhea (12), and vomiting (9). But in the cases presented here, a low intake of potassium in conjunction with the continued excretion of the ion in the urine was an important, if not the principal, factor in producing the cellular deficit.

Excretion of potassium in excess of nitrogen and negative balances of potassium are familiar phenomena in a variety of physiological disturbances. These have been observed in diverse groups of patients maintained on the usual varieties of parenteral fluids and may be accelerated during post-operative periods as part of an injury reaction (31 to 34); they occur during starvation (23) and dehydration (16). The nature of these disturbances suggests that processes of cellular metabolism play an important role in determining the total exchanges of potassium. Our data, however, emphasize the importance of the renal excretion of the ion.

The continued renal excretion of potassium by our patients during periods of low potassium intake does not indicate abnormal renal function, but rather normal function of the kidneys under abnormal circumstances. With one possible exception there was no evidence that any of these patients had diseased kidneys. Their rates of potassium excretion were quite comparable with that of the control subject (R. E.) on a low potassium diet. Furthermore, most patients with severe renal disease have a lower rather than a higher rate of excretion of the ion (35). It is necessary, therefore, to consider the ways in which the abnormal conditions influence the excretion of potassium by the normal kidney.

Winkler and others have demonstrated that under normal conditions increases in the intake of

potassium and in its concentration in serum are associated with increases in the rate of excretion of potassium (27, 28, 36). These findings are confirmed in the control experiments reported here in that large increments of potassium in the urine occurred with slight rises in the serum concentrations. Intermediate analyses of the serum level might have revealed higher concentrations. In the patients during periods of cellular depletion of the ion no positive correlation was apparent between intake of potassium or its concentrations in serum and the daily output of potassium. Rapid uptake of potassium by the cells may have prevented comparable intermediate rises in serum concentrations thus explaining the failure of the kidney to excrete it. But such a simple explanation can hardly account for the fact that in some instances the rate of excretion of potassium was actually lower during periods of administration when the average serum level was normal, than during periods when intake and serum concentration were low. The administration of potassium appeared to shift the equilibria of potassium transfers in the various phases of body fluid from the direction of excretion in the kidneys to movement into tissue cells.

Potassium has been regarded as a "threshold" substance (37). Concentrations of urine potassium below 4 m.eq. per liter have been recorded during diuresis and potassium depletion (11, 28, 38, 39). Concomitant serum concentrations in one study were not greater than those in urine, and in the other studies no concomitant serum concentrations of potassium were reported. In our patients the concentration of potassium was never lower in urine than in serum. Considering renal tubular function as a whole and assuming that the tubular cells are surrounded by the usual type of extracellular fluid, no evidence has been found that potassium can be reabsorbed against a concentration gradient. This does not exclude the possibility that such reabsorption may occur in a given portion of the renal tubule.

Relation of transfers of cellular potassium to those of other ions. Aside from the renal excretion of potassium, it is pertinent to inquire into the relation of transfers of cellular potassium to the exchanges of other extracellular and intracellular ions.

The data confirm previous studies in this laboratory and elsewhere (14) in showing that large

amounts of potassium may enter or leave the intracellular fluid with relatively small increments or decrements of the extracellular fraction of the ion. Some changes in concentration of extracellular potassium may take place. The data show that an abnormally low concentration was always associated with a cellular deficit of the ion. But the converse was not true; one patient, Case 4, (R. W.), had a normal concentration, and one, Case 2, (E. R.), had an elevated concentration of potassium in serum. This is consistent with other investigations in which the evidence suggests that cellular deficiency of potassium may co-exist with normal or elevated concentrations of the ion in the extracellular phase (10, 33, 34). As many patients are suffering from salt loss, dehydration, peripheral vascular collapse and oliguria, it has been suggested that this phenomenon is the result of impairment of renal function. This did not appear to be the fact in Case 4, R. W., but might well explain the high serum concentration observed in Case 2, E. R. This apparent paradox must be kept in mind during any attempt to assess the potassium requirement of the patient.

Under certain circumstances a close relationship has been noted between potassium depletion and alkalosis due to excess of bicarbonate in the extracellular fluid. In four of our six patients the concentration of serum bicarbonate was elevated to some extent before potassium therapy and fell to within normal limits after administration of the ion. Darrow postulated that such a relationship holds only under certain conditions, viz., deficit of only one ion (e.g. potassium), and attainment of a state of equilibrium by adequate renal function (40). All of our cases were studied for periods of 24 hours or longer during which renal function was adequate, but electrolyte deficits were not restricted to only one ion. It is interesting, therefore, that our data fit in as well as they do with Darrow's findings.

One other observation is noteworthy: the elevated serum bicarbonate concentration in Case 1, A. M., did not return to normal after the deficit of cell potassium had been corrected during four days of treatment, but did return to normal three days later when the equivalent amount of sodium had been discharged from the cells. If the change in serum bicarbonate is necessarily related to transfers of either of these cellular cations, this temporal

sequence suggests that it is to the exit of sodium from the cell rather than to the entry of potassium. Such a time lag between the two transfers has been noted in experimental animals by Conway and Hingerty (41). Had the other patients in our series been "saturated" and studied for a longer period of time, reciprocal movements of cellular sodium might have been observed more frequently.

Administration of large quantities of sodium without concomitant use of potassium has also been said to predispose to potassium depletion (42). In four of the patients the intake of sodium, prior to potassium therapy, was low. In none of the cases was depletion of cellular potassium consistently correlated with changes in the concentration of extracellular sodium. It is difficult, therefore, to assess the specific role of sodium therapy, or that of alkalosis, in the cases presented, but certainly deficit of potassium can occur in the absence of either.

Within the limits of the relatively large error of the calculation, a reciprocal relationship between changes in cell sodium and cell potassium was found only occasionally in our studies. Such a relationship appeared to be present in some instances. These results are in agreement with those of Darrow who demonstrated a reciprocal relationship in only three of the six infants with diarrhea whom he treated with potassium (10), and with previous studies in this department (16).

Clinical implications. The subjects of this investigation were probably representative of a large group of patients seen in ordinary hospital practice. All of the patients were maintained on parenteral fluids and were unable to eat. In addition most of them had lost gastrointestinal fluids to some degree. The incidence of unrecognized deficiency of potassium in such patients must be very great.

The diagnosis of potassium deficiency is not simple, as specific signs and symptoms are probably the exception rather than the rule. The diagnosis depends chiefly on recognition of the conditions under which it may develop. The most important of these appears to be a low potassium intake in association with normal renal function. An abnormally low concentration of potassium in serum is strongly suggestive of cellular deficit; a normal or high serum concentration does not ex-

clude it. The most practical proof of the diagnosis at present is demonstration of cellular retention of administered potassium. Other methods include chemical analyses of tissue and possible measurement of total exchangeable potassium by means of the radioactive isotope, K^{42} (43).

Complete elaboration of the contraindications to potassium therapy awaits more extensive clinical experience. In the meantime it is certain that the most important contraindication is inadequate renal function due to any cause (35). Oliguria or anuria caused by intrinsic disease or extrinsic factors are absolute contraindications. As patients who are likely to have potassium deficiency are also likely to be suffering from peripheral vascular collapse, due at least in part to dehydration and sodium depletion, potassium therapy should be deferred until appropriate treatment for these conditions has been instituted. Adrenal cortical insufficiency is a contraindication unless this condition is over-treated with desoxycorticosterone or cortical extract. The limited experience with potassium therapy in patients with organic cardiac disease and associated potassium deficit, permits no comment concerning possible cardiac contraindications (44).

It is not known whether an excessive amount of potassium in the intracellular fluid is deleterious to the organism. But accumulation of potassium in the extracellular fluid is dangerous if it results in an elevation of concentration. The cardiotoxic effects of such an elevation are well known (45 to 49), and may be fatal; other possible toxic effects are less well understood (46). A high concentration of potassium in serum, therefore, contraindicates potassium therapy even though the cells may be depleted of the ion. Evidence has been advanced of increased hazard of potassium administration under certain circumstances when the concentration of serum calcium is low (50).

No arbitrary parenteral dose of potassium can be prescribed. Darrow has estimated that 3.5 m.eq. per kilogram per day of potassium can be administered safely in a period exceeding four to eight hours (51). The subjects of this study received up to 3.7 m.eq. per kilogram per day. The rate of intravenous infusion of the potassium was carefully controlled and not allowed to exceed 20 m.eq. per hour. The concentration of the potassium in the infusion fluids was as high as 70 m.eq. per liter, a concentration which is double that rec-

ommended by Darrow (51). Under these conditions no untoward effects were observed.

No evidence is offered in our data that correction of a deficit of cellular potassium directly benefits the patient. Our patients belonged to no homogenous clinical state with a known case fatality rate or a predictable prognosis. On the other hand, the rationale of replacement therapy has been to restore the patient to a normal chemical state. It appears reasonable, therefore, to attempt to put the replacement of intracellular electrolytes on the same successful basis as that which has been achieved for the constituents of the extracellular fluid.

SUMMARY

Potassium was administered in daily doses of 0.7 to 3.7 m.eq. per kilogram to six adult patients maintained on parenteral fluids. Three normal subjects on isocaloric diets were given 4.4, 3.2, and 0 m.eq. of potassium per kilogram, as controls. The daily exchanges of electrolytes and nitrogen were measured.

In four of the patients the concentrations of potassium in serum were abnormally low. All of the patients retained administered potassium in the cellular phase in excess of nitrogen in amounts varying from 1.2 to 4.6 m.eq. per kilogram. Only one patient received potassium long enough to show that a maximum degree of retention had been reached. Each of the normal subjects who received potassium retained only 0.1 m.eq. per kilogram.

During periods of low potassium intake more potassium was lost in urine than in gastrointestinal fluid. In four patients during such periods the quantity in urine was greater than was the quantity during periods when the intakes were high and concentrations in serum were normal. The minimum amounts in urine in three of the patients deprived of exogenous potassium and maintained in nitrogen equilibrium, were 28, 27 and 6 m.eq. per day, and in the normal subject 34 m.eq. per day. The potassium U/P ratio was never less than 1.0.

CONCLUSIONS

1. Adult patients deprived of potassium by gastrointestinal disorders retain significant amounts of potassium when administered.

2. Retention of potassium probably indicates cellular deficit of the ion.

3. Such deficits are frequently but not always associated with abnormally low concentrations of potassium in serum.

4. During administration of potassium to such patients the excretion of potassium tends to decrease rather than to increase.

5. The kidney in normal subjects as well as in depleted patients, under conditions of maximal need for conservation, has a limited minimal rate of excretion of the ion. This continued excretion by the kidney is an important factor in producing a state of potassium deficiency.

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CAN VASCULAR SHUNTING BE INDUCED IN THE KIDNEY BY VASOACTIVE DRUGS?¹

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Modern concepts of renal physiology are based on the assumption that, at least in normal subjects without impotent or aglomerular tubules, nearly all the blood goes first through the glomeruli before reaching the peritubular capillaries (1). Therefore, blood supplying the tubular system is thought to come entirely from the efferent arterioles. Anatomists have described almost every kind of anastomosis, however, which could shunt various parts of the functioning parenchyma (2). Except by Spanner (3), little consideration has been given to their possible significance in the normal kidney (4-6). Likewise, very little is known of the role of renal lymphatic vessels in the function of the kidneys (7, 8).

The recent work of Trueta, *et al.* (9) aroused general interest in this subject, because for the first time³ an attempt was made to demonstrate the importance of renal vascular channels which bypass the cortex. Although these authors offer only morphological evidence and employ rather vigorous methods (crushing a limb, very large doses of epinephrine, etc.), they were able to show under these conditions a shift between cortical and medullary blood flow in the rabbit. Therefore, it seemed rewarding to investigate whether a similar phenomenon occurs in human subjects and dogs under normal or pathological circumstances, and whether, as Trueta indicated, this is related to the presence of arterial hypertension. The experiments here reported were made in order to detect, if possible, any changes in the relative blood supply of the different parts of the nephron after administration of certain drugs in pharmacologically active

doses. It was believed that the opening or closing of anastomotic channels between arteries and veins would be reflected by relative changes in the renal extraction of p-amino hippurate and/or mannitol, the utilization of oxygen, or both.

METHODS

Catheterization of the right renal vein was performed in 14 patients using the technique described by other authors (11, 12). Eight suffered from arterial hypertension and six did not. Catheters Nos. F-8, -9, or -10 were used. In four of the earlier experiments a single intravenous injection of sodium para-amino hippurate (PAH) and mannitol solutions was given as soon as the position of the catheter had been checked by roentgenograms. The concentration of the solutions was chosen so as to provide acceptable plasma levels during one hour or longer. In later experiments, a continuous infusion of PAH and mannitol was used instead of the single injection in order to provide relatively constant levels of these substances in the blood. After one, two or three simultaneous samples from the femoral artery or antecubital vein and from the right renal vein had been drawn, epinephrine or histamine was given subcutaneously, and two or three more simultaneous blood samples collected three to 45 minutes later. In one case pentothal-sodium in anesthetic dose was injected intravenously. In all experiments PAH and mannitol were determined in renal and peripheral blood. In six the oxygen content and in four the oxygen capacity and the hematocrit were also measured. All blood samples were centrifuged as soon as possible in order to avoid diffusion of PAH from red blood cells. PAH and mannitol were measured by the method of Goldring and Chasis (13) modified only as regards the technique for estimating the plasma and urine mannitol "blank" (14). The oxygen content and capacity were determined by the method of Van Slyke and Neill (15). Hematocrits were read in Wintrobe tubes. The blood pressure was measured by the auscultatory method.

In 15 dogs blood from the renal vein and from the renal artery and/or femoral artery was drawn slowly and more or less simultaneously after the vessels had been exposed under nembutal anesthesia. For technical reasons, only partial experiments could usually be performed due to the large amounts of blood necessary for complete analyses. In some dogs the arterio-venous oxygen difference and the hematocrit, and in others, the

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² Fellow of Swiss Foundation for Medical and Biological Research.

³ Actually, several years previously, Fuchs and Popper (10) dealt with the problem of the "medullary" circulation, but their evidence, based upon the separation of India ink during diuresis, was not too convincing.

TABLE I
Changes in arterio-renal venous differences after intravenous injection of epinephrine in dogs

Dog No.	Dose	Time after injection	Oxygen content FA-RV	Hemo- concentration RV-FA RV	Hemo- concentration RV-RA RV	Oxygen capacity RA-RV	Plasma proteins RV	PAH† extraction	PAH† clearance
	mgm.	min.	vol. %	%	%	vol. %	gm. %	%	cc./min.
19	0.1	0 5 16.5	3.20 2.00 4.80						
20	0.1	0 2 8	3.70 1.20 2.00						
22	0.1	0 3 10	1.95 1.40 2.55						
29	0.1	0 8 20	2.45 1.60 2.70					85.0 29.0	90.0 61.2 10.8
102	0.1	0 6 15	1.50 1.85 2.20					70.0 85.0 82.5	84.8 84.5 51.2
105	0.1	0 7 32	1.80 2.10	11.1 7.5				83.0 76.7 61.5	
106	0.2	0 3 12	2.40 1.85 2.95	-0.1 16.3 -2.7		4.9*	7.08 7.50		
110	0.2	0 2			4.8 9.0	-1.1			
112	0.15	0 3.5	1.30 1.90	1.8 0	5.0				
116	0.2 500§	0 2.5 10			3.3 13.1 1.8	-0.5 -3.1			
117	0.2 500§	0 3 10			2.5 4.0 1.0				
205	0.25	0 4			1.6 10.8				
217	0.2	0 4 28		0.1 5.0 5.8				68.5 48.0 65.0	
221	0.1	0 5 28		-8.8 -2.2 -3.6				57.0 66.5 64.0	
231	0.2	0 3		6.8 3.9	6.75 7.95		4.67 5.03		

Note: FA indicates sample taken from femoral artery, RA from renal artery, and RV from renal vein.

* FA-RV.

† FA-RV.

‡ FA.

§ 15-minute periods.

§ Caffeine.

TABLE II

Oxygen content, hematocrit, and corrected oxygen content in dog experiment No. 106

Condition	O ₂ Content			Hematocrit			Corrected O ₂ content *		
	Femoral artery	Renal vein	A-V diff.	Femoral artery	Renal vein	Hemo-conc.	Femoral artery	Renal vein	A-V diff.
Control	vol. %	vol. %	vol. %	%	%	%	vol. %	vol. %	vol. %
3 minutes after epinephrine 0.2 mgm.	18.80	16.40	2.40	54.75	54.10	-1.1	18.80	16.60	2.20
12 minutes after epinephrine	20.00	18.15	1.85	57.10	68.20	+16.5	19.20	14.60	4.70
	18.15	15.20	2.95	53.90	52.50	-2.6	18.40	15.80	2.60

* Corrected to a constant hematocrit of 54.75.

PAH extraction,⁴ were determined before and after the intravenous injection of epinephrine (50 to 200 gamma) using the methods described above.

RESULTS

a. *Experiments in dogs (Tables I, II).* After epinephrine was injected the difference between the oxygen content in femoral arterial and renal

⁴ The "extraction" is defined by the expression $\frac{P-R}{P}$, where P is the concentration of the substance in the peripheral and R in the renal venous plasma.

venous blood was lower in five dogs (Nos. 19, 20, 22, 29, and 106), and higher in three (Nos. 102, 105 and 112). The reaction to the drug was minimal in the latter (Table I). Renal arterial hematocrit values were consistently lower than venous, and the usual rise occurring after epinephrine was less marked than in renal venous blood. Femoral arterial hematocrit values were not consistent, being sometimes higher and sometimes lower than those from renal arterial blood. Epinephrine produced less hemoconcentration in fem-

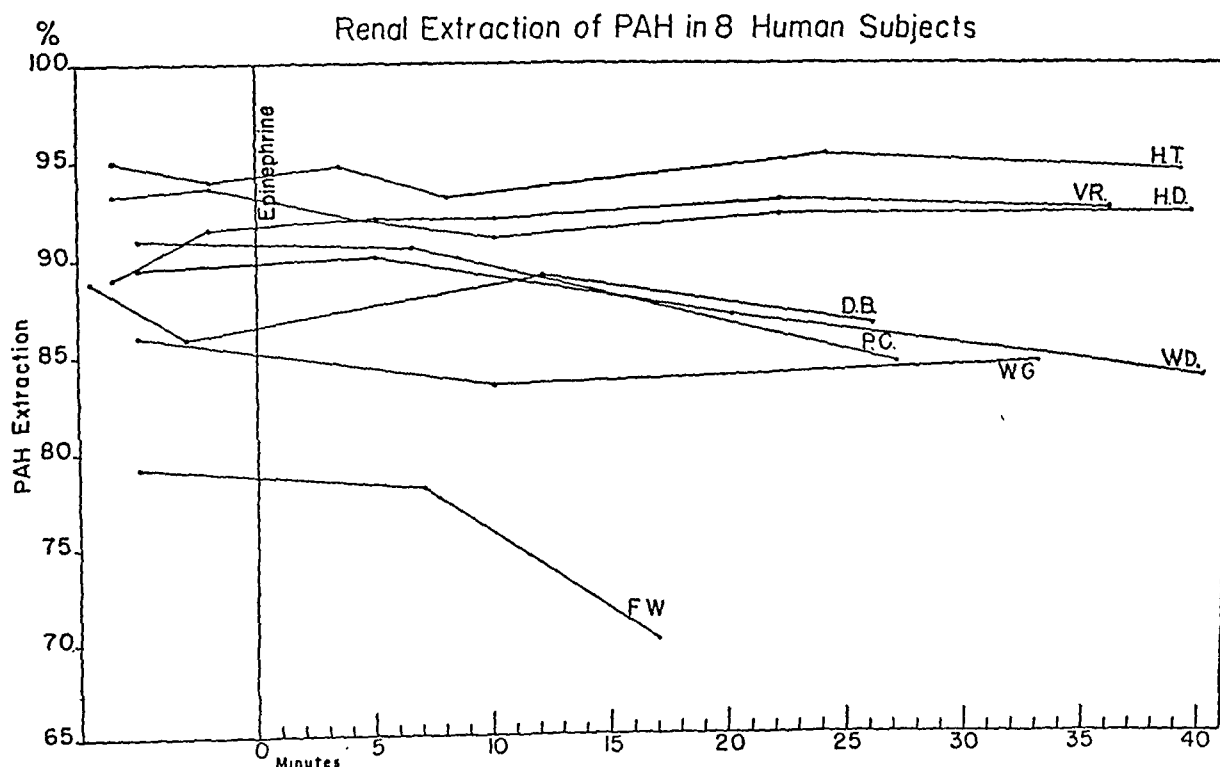


FIG. 1. VARIATIONS IN THE EXTRACTION OF PARA-AMINO HIPPURATE BY THE RIGHT KIDNEY AFTER EPINEPHRINE WAS INJECTED SUBCUTANEOUSLY

The control values to the left of the vertical line were taken at least ten minutes apart, and after sufficient time had elapsed to allow the blood levels to come to equilibrium. The vertical line indicates the time of injection of epinephrine. Except for three cases, there is no significant change in the extraction of PAH (see Table III). Note that the reduction occurs only in the last determination. Patients F. W. and W. D. suffered from arterial hypertension, as did D. B. and V. R.

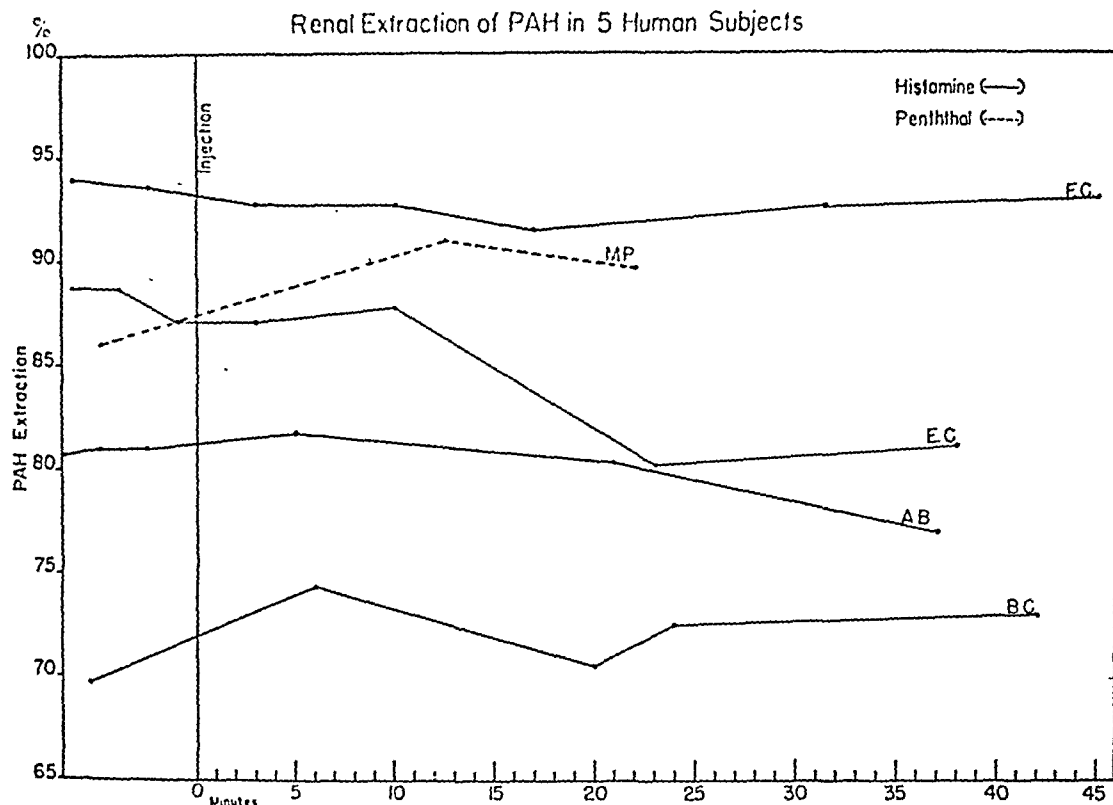


FIG. 2. VARIATIONS IN THE RIGHT RENAL EXTRACTION OF PAH AFTER THE SUBCUTANEOUS INJECTION OF HISTAMINE (—) AND THE INDUCTION OF PENTOTHAL ANAESTHESIA (---)

All patients were hypertensive but F. C. The vertical line indicates the time of injection. Note that a significant decrease occurred only in one case (E. C.), and this 23 minutes after injection.

oral arterial blood than in that from the renal vein, except in one dog (No. 231).

Changes in the oxygen capacity paralleled the hematocrit values, and the same is roughly true with regard to the plasma proteins, indicating that actual hemoconcentration occurred. Caffeine in two experiments appeared to lessen the arterio-venous hematocrit difference. The extraction of PAH by one kidney was influenced by intravenous epinephrine in four of five dogs, but the direction of the change was inconsistent (Table I). In one (No. 105) it decreased progressively, in one (No. 217) temporarily, and in two it rose. One experiment indicated a pronounced fall (No. 29) but the blood sample may have become contaminated with ureteral urine.

b. *Experiments in human subjects (Table III).* Fourteen successful experiments were completed. Renal arterio-venous differences in oxygen content were determined in six patients. After pentothal anesthesia was given in one (M. P.) the difference was slightly increased. When epinephrine

was injected it became decreased in two (W. G. and F. W.), but renal venous blood became more concentrated as evidenced by plasma protein levels. In two other subjects (P. C. and W. D.) the arterio-venous oxygen difference was increased. It was probably unchanged in patient D. B., although the second arterial determination of oxygen was not entirely reliable, a small clot having been found in the syringe. Hematocrit values, determined in four cases, were increased in two by epinephrine and in one (W. D.), this was greater in renal venous than in femoral blood. They were unchanged during pentothal anesthesia in one, and slightly increased in one after histamine was injected.

The renal extraction of PAH became decreased 16 to 40 minutes after epinephrine was injected in three subjects. This decrease ranged from 91.0 to 84.5 per cent in subject P. C., from 79.7 to 69.6 in F. W., and from 89.5 to 83.7 in W. D. (Figure 1). No significant changes were observed in six patients. After histamine was given, changes occurred in two (E. C. and A. B.), where the ex-

traction fell from 87.1 to 80.2 and from 80.7 to 77.0 respectively (Figure 2).

The mannitol extraction usually increased shortly after epinephrine was injected, suggesting that efferent arteriolar constriction occurred, returning to its previous level later (except in case F. W. where it was definitely lower in the last period). After histamine was given, it increased in three cases, and markedly decreased in one (E. C.). Variations in the one experiment during which pentothal anesthesia was induced were not consistent.

In some of these patients urine was collected during the renal venous catheterization, and the urinary excretion of mannitol and PAH was compared with their renal extraction. A discrepancy between the plasma flows calculated from C_M/E_M and C_{PAH}/E_{PAH} was found (16). This discrepancy cannot be explained by the presence of arteriovenous shunts, as the extraction percentages of these materials were not unduly altered. These results will be reported elsewhere (17).

DISCUSSION

It is conceivable that shunts of several types exist in the kidney. It could reasonably be expected that our experiments would be capable of demonstrating at least three of them, if they were physiologically important and responded to ordinary doses of vasoactive drugs, namely:

1. *Direct arterio-venous anastomoses between relatively large vessels (arcuate or interlobular), arteriola rectae, "juxta-medullary by-passes"* (9, 18), which short-circuit both glomeruli and tubules. Opening of these shunts would decrease PAH and mannitol extractions and renal arteriovenous oxygen differences.⁵ This was not found to be the case.

⁵ It is possible that "juxta-medullary by-passes" resulting from blood flowing through nephrons in this region, produce about the same effect as direct arteriovenous shunts. It is not likely that glomeruli, which have efferent arterioles as large as afferent (9), perform much filtration, and therefore little mannitol would be filtered. Even if the tubules corresponding to these glomeruli are functioning, relatively small amounts of PAH will be extracted from the blood by the tubular cells, unless there is an adequate intratubular flow of urine. Moreover, the proportion of juxta-medullary nephrons to cortical ones is probably not greater than 10 per cent (18). Therefore, if these nephrons should become

2. *Vessels supplying the tubules by-passing the glomeruli.* Opening of these would decrease the mannitol extraction alone, PAH and oxygen differences remaining unchanged. The same effect would be produced by the presence of "aglomerular tubules" (1). Changes in the mannitol extraction, however, were not consistent.

3. *Direct communications between efferent glomerular arterioles and renal veins by-passing the peritubular capillaries.* Opening of such anastomoses would decrease the PAH extraction and decrease slightly the oxygen differences, mannitol extraction being unchanged. One would expect the same from "impotent nephrons" (1). Extractions of PAH, however, remained relatively constant in most cases.

It is also conceivable that some of the substances entering the kidney leave it via lymphatic vessels and by-pass the renal vein either after being reabsorbed, or after interstitial diffusion. Unfortunately, there is at present no innocuous way to demonstrate such a mechanism in man.

The first type of shunt should be easily demonstrable and would probably be the most important in physiopathological conditions of the kidney. There is some evidence that such shunts exist, at least in animals. Trueta claims to have seen "red blood" in the renal veins of rabbits (9). We have observed it in dogs only during trauma to the kidney at operation. Unexplained low PAH or diodrast extractions have been observed both in the dog and in man (12, 19, 20). The existence of shunts has been postulated in order to explain "reflex anuria" (21), and the "crush syndrome" (9). These observations have been made usually under experimental, operative or severe pathological conditions. The importance of anastomoses would be greater if they were concerned with ischemia of certain parts of the kidney, and if relatively mild stimuli (nervousness, autonomic instability, emotional factors, pain) would be able to shift the cortical circulation to a medullary one. For that reason, epinephrine was used in our experiments. Histamine was employed as well, because a renal action somewhat similar to that of epinephrine has been demonstrated (22).

functional to any great extent, at the expense of the cortex, one might expect a considerable depression of the extraction of both mannitol and PAH.

When renal arterio-venous oxygen differences were measured in the dog, Trueta's findings appeared to be confirmed, *i.e.*, the oxygen content in renal venous blood increased after the intravenous injection of epinephrine. However, when hematocrit, oxygen capacity and plasma proteins were also determined, (Table I), it appeared that this increase was simulated by a marked hemoconcentration (according to oxygen capacity and hematocrit), of as much as 22.2 per cent in the renal blood, and of only 4.4 per cent in the peripheral blood in one case (Figure 3). The increase in plasma proteins was not as striking. When arterio-venous differences were corrected to a constant oxygen capacity, epinephrine appeared to cause an increase (from 2.2 to 4.7 volumes per cent). We observed the same increase in experiments in which mild reactions to epinephrine were elicited and negligible hemoconcentration occurred (Nos. 102, 105, and 112).

However, only renal venous and femoral arterial blood were compared. According to Dole, *et al.* (23) unexplained differences in the oxygen capacity of renal and peripheral blood can be observed in the dog. Therefore, in later experiments samples were drawn simultaneously from the renal vein, renal artery, and sometimes from the femoral artery. At variance with the assumption of Dole, it was found that there was a consistent difference between venous and arterial renal hematocrits (and oxygen capacities), the former being more concentrated. This difference was increased by epinephrine and decreased by caffeine (Nos. 116 and 117). This hemoconcentration cannot be entirely explained by the amount of excreted urine. With a diuresis of 0.5 cc. per minute, the expected hemoconcentration would be only 0.5 per cent. Even if epinephrine reduces renal blood flow more than urine flow, hemoconcentration greater than 2 or 3 per cent is not likely. Therefore, some loss

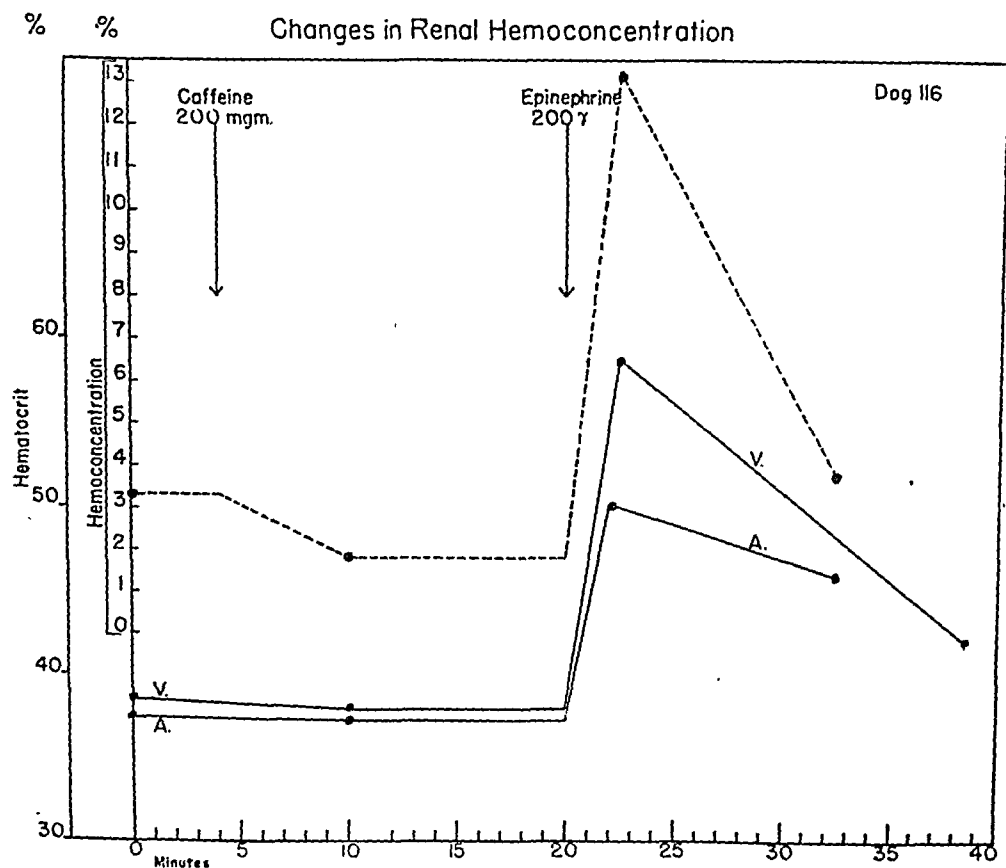


FIG. 3. CHANGES IN THE HEMATOCRIT (—) AND HEMOCONCENTRATION (---) IN ONE DOG FOLLOWING THE INTRAVENOUS INJECTION OF CAFFEINE AND EPINEPHRINE

V refers to that in the renal venous, A to that in the renal arterial blood. Note the rise immediately after epinephrine was given.

TABLE III—Continued

Subject	Drug and dose	Time after injection	Oxygen content FA—RV	PAH content P—RV	PAH content Extrac-tion†	Mannitol content P—RV	Mannitol content Extrac-tion†	Plasma proteins RV	Hemato-crit RV—FA	Diagnosis
	mgm.	min.	vol. %	mgm. %	%	mgm. %	%	gm. %	%	
H. D. ♂	0.7 E	0		2.88	93.3					Peptic ulcer
		0		3.08	93.6	11	10.0			
		4		3.44	92.0	13	11.5			
		10		3.59	90.9	15	13.3			
		22		3.59	92.3	17	14.8			
		40		4.09	92.4					
H. T. ♂	0.8 E	0		1.34	95.0	*2.18†	16.2			Primary syphilis
		0		1.41	94.0	2.56	20.1			
		3.5		1.76	94.7	3.07	25.9			
		8		1.79	93.2	3.48	28.8			
		24		2.04	95.3	3.08	27.5			
		39		2.06	94.5	3.06	26.7			
E. C. ♀	0.5 H	0		4.05	88.7	29	20.8			Hypertension
		0		4.02	88.7	30	21.5			
		0		4.08	87.1	33	23.7			
		3		4.14	87.1	29.5	20.9			
		10		4.38	87.8	17.5	11.8			
		23		3.81	80.2	10.0	6.2			
F. C. ♂	0.4 H	38		3.88	81.1	23.0	14.1			Peptic ulcer, Parkinsonism
		0		2.27	94.0	18.9	18.9	5.47		
		0		2.24	93.8	16.2	16.2	(-0.28)§		
		3		2.24	92.7	22.2	22.7			
		9		2.36	92.7	17.4	18.8			
		17		2.27	91.3	13.1	14.4			
B. C. ♂	0.5 H	31		2.10	92.6	11.9	13.3			Hypertension
		45		2.30	93.0	14.5	16.3			
		0		2.19	69.8	14	11.7		-.70	
		6		2.43	74.3	16	13.1		-.40	
		20		2.19	70.5	15	12.6		-.30	
		24		2.22	72.5	14	11.9			
A. B. ♂	0.5 H	42		2.10	73.0	20	17.4			Hypertension
		0		3.97	80.7	21	15.3	5.95		
		0		4.00	81.0	18	13.4	(0.00)§		
		0		3.90	81.2	23	16.9			
		5		4.21	81.8	26	19.0			
		21		4.29	80.2	27	19.0			
*M. P. ♀	P	37		3.84	77.0	23.5	16.9			Hypertension
		0	1.8	2.30	85.8	20	15.6		.30	
		0		0.59	100 ?	16.3	22.5			
		12-14	2.25	3.96	91.5	28.0	16.8			
		22		2.68	89.9	33.0	21.7			

Note: FA indicates sample taken from femoral artery, RV from renal vein and P from either femoral artery or antecubital vein. All drugs except pentothal were given subcutaneously. E=epinephrine; H=histamine; P=pentothal.

* In these cases one single injection of PAH and mannitol was given except for M. P. who was given two injections. In the remainder constant infusions were employed.

† P—RV
P

‡ Sodium thiosulfate used instead of mannitol.

§ RV—FA.

femoral and renal venous blood were compared. In the dog there may be a discrepancy between femoral and renal arterial samples. At any rate, under control conditions, there were no significant differences between femoral arterial and renal venous hematocrits in man.

In the evaluation of renal arterio-venous oxygen differences one point must be emphasized. Reduction of blood flow may decrease greatly the oxygen content of the venous blood and partial shunting may partly compensate for this change. For that reason, unless opening of large shunts

occurs, these determinations are of less value than PAH and mannitol extractions.

Changes in PAH extractions in dogs were not very consistent after epinephrine was given, and the results are not conclusive. In one case (No. 29) it fell to a very low value, but errors in technique cannot be excluded. Except for this single questionable case, there appeared no changes consistent with the opening of shunts. The results of human experiments are certainly less questionable, and due to the standard conditions under which the tests were performed, even slight variations can be considered significant. It may be assumed that partial shunting of the first type was produced by epinephrine in case F.W. (Table III), as both the PAH and the mannitol extractions were lower than the control values (11.4 and 33.3 per cent respectively). In cases P. C. and W.D., where the PAH extraction was reduced 7.1 and 6.7 per cent, the mannitol extraction was not affected, so that shunting might have excluded mainly the peritubular capillaries (type of shunting No. 3). Impairment of tubular excretory capacity due to anoxia, however, could account for this reduction (26).

Pentothal anesthesia was without visible effect in one case, and histamine may have opened shunts of the first type in one (E. C.) and of the third type in another (A. B.). At any rate, the greatest shunting elicited in one case where the reaction to epinephrine was severe, was not much more than one-tenth of the total renal blood flow. Moreover, this phenomenon was observed in a diseased kidney where the extraction was low at the beginning. Although four out of the five patients in whom changes occurred had hypertension, our findings do not support the hypothesis of Trueta, *et al.* that large shunts may open under physiological conditions and be responsible for intermittent cortical ischemia concerned in hypertensive disease. Bradley and Bradley (27) were unable to demonstrate appreciable reduction in PAH extraction after abdominal compression in eight normal subjects, although renal blood flow was reduced. While a persistently low PAH extraction by damaged hypertensive kidneys could be produced by permanent shunting, tubular impairment and the destruction of active parenchyma with subsequent replacement by fibrous tissue seems a more likely explanation. Shunts of that kind would not have

any etiologic significance, being the result and not the cause of the renal alterations.

Although these experiments do not disprove the presence of arterio-venous by-passes in the kidney of man and of the dog, they do indicate that after doses of epinephrine or histamine large enough to cause systemic reactions, these vascular channels do not consistently appear. When they do, they are not very important. In the dog, furthermore, they were not demonstrated even after large doses of epinephrine. It is still possible that such channels may become functional following toxic doses of drugs, during severe shock, or after crushing wounds. Their significance in minor physiological alterations in homeostasis is therefore questionable.

SUMMARY AND CONCLUSIONS

1. Experiments involving determinations of PAH, mannitol, oxygen content and hematocrit in renal venous and peripheral (or renal) arterial blood before and after injection of epinephrine, histamine or pentothal were performed in eight hypertensive and six normotensive patients, and in 15 dogs. In patients, renal venous blood was collected by catheterization of the right renal vein.

2. The maximal decrease in the extraction of PAH by the kidneys of patients after subcutaneous injection of epinephrine or histamine was 11.4 per cent; dog experiments were inconclusive.

3. In two human subjects, arterio-venous oxygen differences were increased by epinephrine, and in three, decreased.

4. In dogs, there was a consistent difference in the oxygen capacity, hematocrit, and plasma protein content between renal arterial and renal venous blood, the latter being more concentrated. This difference was increased by epinephrine. It is suggested that this loss of fluid from the renal blood may have occurred through renal lymphatic vessels or other vascular channels by-passing the renal vein.

5. From these experiments it can be concluded that large intermittent renal shunts can not be elicited in man by physiological doses, or in dogs by larger doses, of epinephrine. Even in subjects with essential hypertension, histamine or epinephrine produced relatively small and inconsistent changes which could be ascribed to the presence of shunts.

ACKNOWLEDGMENTS

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ELECTROPHRENIC RESPIRATION. II. ITS USE IN MAN¹

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In a previous investigation it was shown that artificial respiration could be effectively administered to the cat, dog, monkey and rabbit in the absence of spontaneous respiration by electrical stimulation of one or both phrenic nerves (1, 2). During the development of the applications of the technique, an opportunity occurred for testing the method in man. A patient with severe chronic, diaphragmatic flutter was operated on for the purpose of 1) crushing the left phrenic nerve and 2) temporarily blocking the right phrenic with procaine. At the time of operation a single, slender, multiple-strand, plastic-covered wire was spirally wound around the right phrenic nerve in its exposed portion and the other end of the lead wire was brought out through a simple needle puncture wound about 2 cm. lateral to the site of incision. The operation was performed under local anesthesia. Five hours later, electrophrenic respiration was begun.

The method is described in detail in a previous communication (2). Briefly, it is the following. An ECG plate secured at the wrist functions as an indifferent electrode for the single stimulating electrode on the phrenic nerve. The stimulating current is derived from a Grass stimulator set for a frequency of 40/sec. and an impulse duration of 2 milliseconds. The current is run through a rotating potentiometer which rhythmically varies the voltage in such a way as to cause the diaphragm to perform a gradual contraction and relaxation similar to that which it performs during spontaneous breathing.

Air flow patterns were obtained with the pneumotachograph developed by Silverman (3) and Silverman and Whittenberger (4). From the pneumotachogram one is able to derive information related not only to the rate and contour of the respiratory pattern but also precise values for the patient's ventilation volume. The level of arterial oxygen saturation was observed throughout the period of phrenic nerve stimulation by means of the Millikan oximeter (5).

The experiments on this patient were designed to achieve five objectives:

1. To ascertain whether electrophrenic respiration could maintain minute volumes equal to or

greater than the spontaneous resting minute volume with submaximal stimulation of one phrenic nerve.

2. To ascertain the ease with which a smooth diaphragmatic motion can be obtained in man.

3. To find out if the unanesthetized patient would cease spontaneous respiratory activity when adequate aeration was supplied by means of phrenic stimulation (as was previously found to be the case in the experimental animal under anesthesia).

4. To estimate the amount of pain or discomfort produced by effective electrical stimulation of the phrenic nerve.

5. To ascertain whether adequate arterial oxygen saturation could be maintained with the submaximal stimulation of one phrenic nerve.

RESULTS

1. Figure 1 shows the pneumotachograms of A) spontaneous respiration and B) electrophrenic respiration. Table I compares values obtained

TABLE I
*Respiratory measurements during spontaneous breathing
and electrophrenic respiration*

	Spon- taneous respira- tion	Electro- phrenic respira- tion
Rate per minute	26.6	23.3
Minute volume in liters	5.18	7.52
Tidal volume in liters	0.195	0.323
Average flow rate during inspiration, in liters per minute	12.0	15.9
Ratio of time of inspiration to time of total respiratory cycle, in per cent	43.1	47.3

during spontaneous respiration and electrophrenic respiration for respiratory rate, minute and tidal volumes, average flow rate during inspiration, and the fraction of the respiratory cycle taken by the inspiratory phase. These results were obtained at a rate of artificial respiration slightly slower than the patient's spontaneous rate and with submaximal stimulation of one phrenic nerve. It can be

¹ Aided by a grant from The National Foundation for Infantile Paralysis.

seen that an exchange of air significantly larger than the patient's spontaneous exchange is readily achieved at a slower than the spontaneous rate. A still larger exchange of air resulted from maximal stimulation. It was established in this patient, as in the experimental animal, that the depth of respiration is proportional to the peak voltage applied. The regularity of the tracing in Figure 1B indicates that the patient was not contributing to the respiratory effort. This phenomenon will be discussed later.

2. It is apparent from Figure 1B that the diaphragmatic contraction produced by electrical stimulation of the phrenic nerve in this patient was a smooth motion. (The minor irregularities during diaphragmatic relaxation in the record are due to the diaphragmatic flutter.) A smooth inspiratory curve was, in fact, predicted from the theoretical basis on which electrophrenic respiration is based; namely, that increasingly forceful contraction of the diaphragm results from the spread of current to include more and more fibers of the phrenic nerve as the voltage is increased. Since the phrenic nerve in man is a single trunk and is considerably larger than that of the experimental animal, a graded voltage spread is more easily and smoothly obtained. As observed visually, the movement of

the right diaphragm resulting from phrenic nerve stimulation closely resembled that seen during spontaneous respiration.

3. It was apparent from simple observation of the patient that during artificial respiration there was no respiratory effort other than that of the right diaphragm, which resulted from electrical stimulation. In order to obtain graphic evidence of this fact, two additional maneuvers were carried out. Figure 2 is a pneumotachogram of spontaneous respiration interrupted by the onset of electrical artificial respiration. There is no evidence that spontaneous respiratory activity occurred after phrenic nerve stimulation had been started. Spontaneous activity would be evident as irregularities in the pattern. The reverse sequence was employed to find out whether, upon abruptly terminating artificial respiration, the lack of spontaneous respiratory activity would be apparent from the lack of air flow as registered on the pneumotachogram. Figure 3 is the result. Artificial respiration was stopped at the signal and it can be seen that no respiratory activity was present until the apnea had persisted for over 16 seconds. Breathing then returned with a gradual increase in amplitude until it regained its pre-stimulus contours. This interesting observation, namely, that

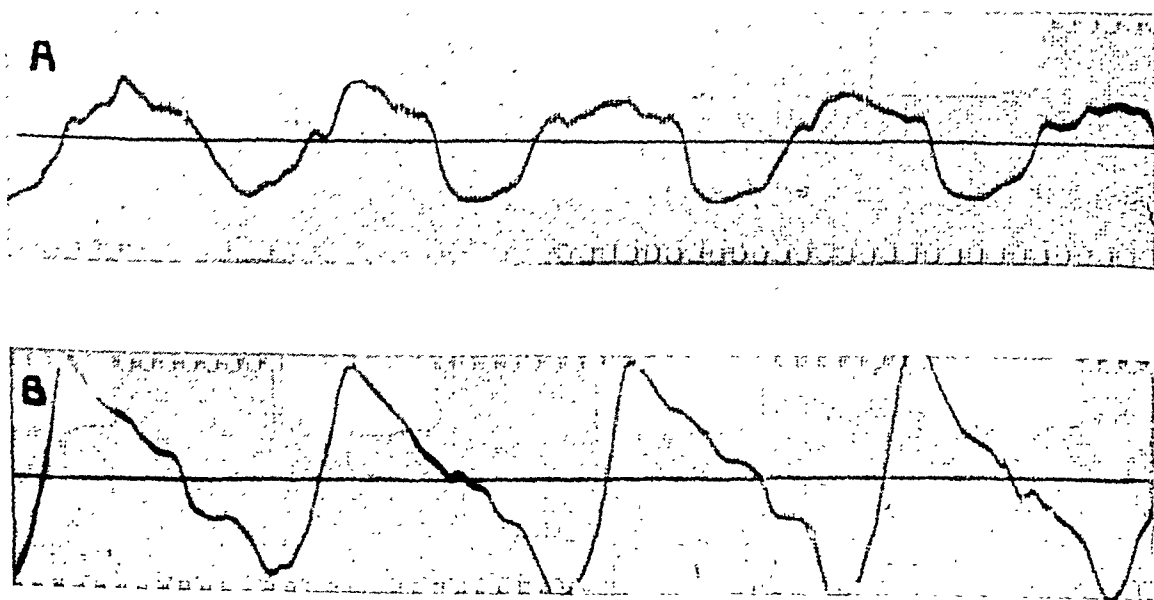


FIG. 1. A, PNEUMOTACHOGRAM OF SPONTANEOUS RESPIRATION; B, PNEUMOTACHOGRAM DURING ELECTROPHRENIC RESPIRATION

Area under the curve represents volume of air flow during inspiration; that above the base line represents expiration. The area is directly proportional to the volume of air flow.

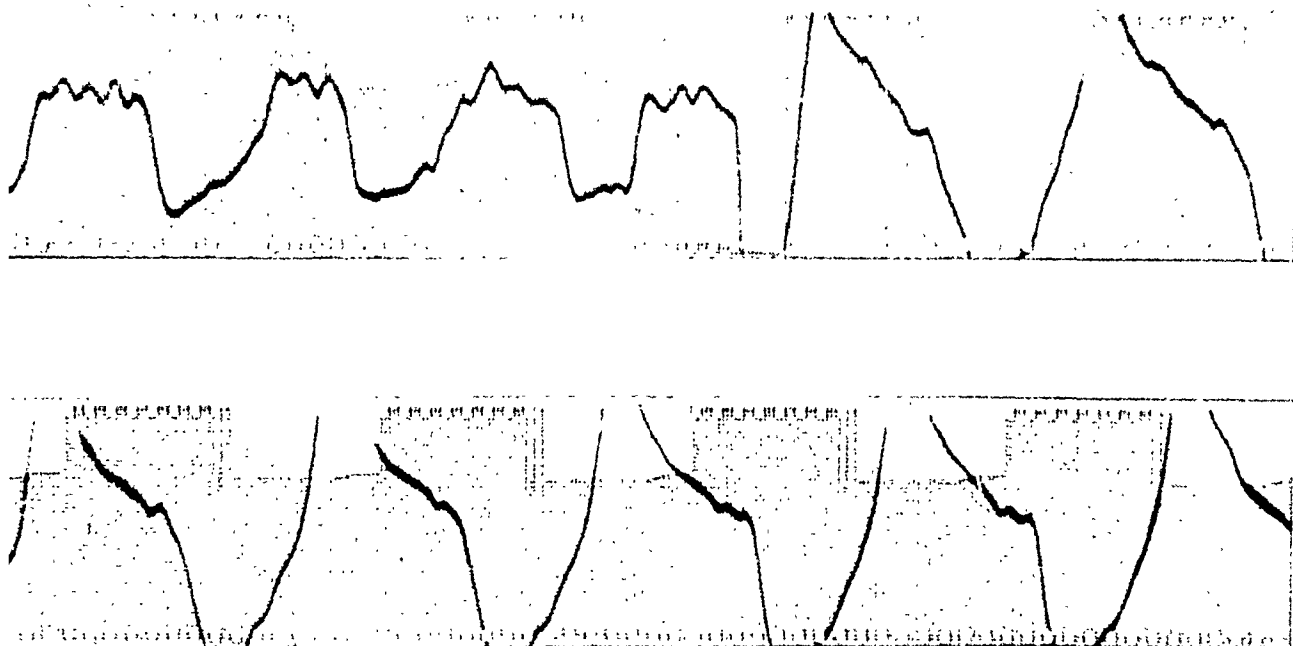


FIG. 2. PNEUMOTACHOGRAM OF SPONTANEOUS RESPIRATION INTERRUPTED BY THE ONSET OF ELECTROPHRENIC RESPIRATION

Phrenic nerve stimulation was started at the signal. The two strips are continuous.

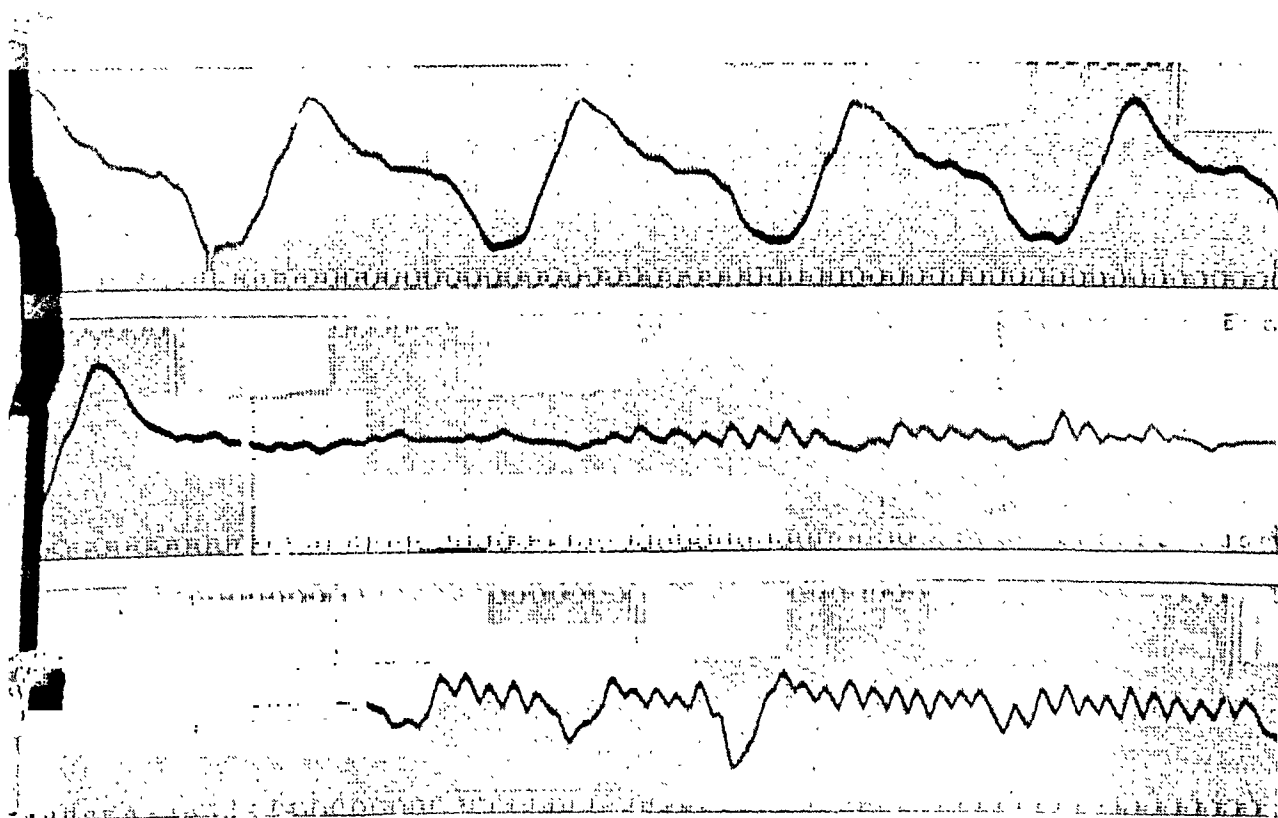


FIG. 3. APNEA INDUCED BY ELECTROPHRENIC RESPIRATION

The patient was on artificial respiration at the start of this pneumotachogram. At the signal, phrenic stimulation was discontinued and the tracing shows the absence of spontaneous respiratory effort during the period immediately following cessation of artificial respiration. The minor irregularities are due to the diaphragmatic flutter. The three strips are continuous.

the individual completely relinquishes spontaneous control of respiration while on electrophrenic respiration, is not yet fully explained. Animal experiments in which blood gas tensions were observed indicate that the prompt cessation of spontaneous respiration is not due to a fall in carbon dioxide brought about by overventilation. The neural pathways involved in this phenomenon are being investigated.

4. The evaluation of pain in this patient was not easy. During phrenic nerve stimulation the patient experienced discomfort referred to the right shoulder along the ridge of the trapezius muscle. This information was obtained only on direct questioning, was never volunteered. The patient did not appear to be in pain and fell asleep several times during the longer periods of artificial respiration. Conclusions as to the degree and importance of the intensity of the discomfort produced will have to be deferred until a variety of patients have been studied.

5. It was thought, prior to our experience with this patient, that the relatively stable mediastinum of the human subject might prevent the adequate aeration of the contralateral lung when only one phrenic nerve was stimulated, even though reasonable respiratory minute volumes were achieved. In order to obtain evidence in this matter, arterial oxygen saturation (by the oximeter method) was observed during spontaneous respiration and during artificial respiration in the absence of spon-

taneous respiratory activity. Figure 4 shows the results. It is clear that a small but definite rise in arterial saturation occurred within 60 seconds of the onset of electrophrenic respiration. A rise of 1 per cent in saturation at the upper range of the oxyhemoglobin dissociation curve indicates a rise of 10 mm. Hg or more in the mean effective alveolar partial pressure of oxygen. This signifies an improvement in lung ventilation out of proportion to a 1 per cent rise in oxygen saturation. The oximeter was calibrated by having the patient inhale 99.6 per cent oxygen.

Artificial respiration by phrenic nerve stimulation was induced in this patient for several periods varying in length from five to 76 minutes on two occasions during the five days following electrode application. The procedure apparently resulted in no functional damage to the nerve, since fluoroscopic examination of spontaneous diaphragmatic activity on the day of electrode removal revealed excursions equal in amplitude to those observed before the experiment.

CONCLUSIONS

1. Artificial respiration by phrenic nerve stimulation can be performed in man.
2. A smooth, gradual, diaphragmatic contraction occurs when an increasing voltage is applied to the phrenic nerve. The diaphragm thus performs a motion closely resembling that which performs during natural inspiration.
3. Respiratory minute volumes in excess of patient's spontaneous minute volumes can readily be obtained with the submaximal stimulation of one phrenic nerve.
4. The depth of respiration is proportional to the peak voltage applied to the phrenic nerve man as in the experimental animal.
5. Adequate oxygenation of the blood can be maintained by electrophrenic respiration in the absence of spontaneous respiration.
6. The human subject, like the experimental animal, completely relinquishes spontaneous control of respiration when electrophrenic respiration is induced.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Dr. K. Emerson, J. L. Blodgett, and Lewis Dexter for opportunity of studying this patient.

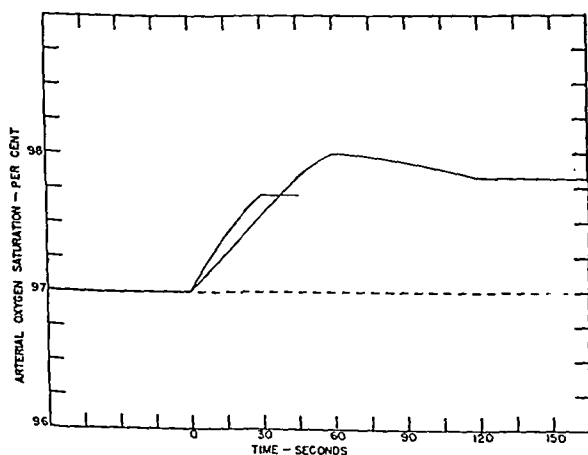


FIG. 4. EFFECT OF ELECTROPHRENIC RESPIRATION ON ARTERIAL OXYGEN SATURATION IN THE ABSENCE OF SPONTANEOUS RESPIRATORY ACTIVITY

The patient breathed throughout.

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THE VOLUME OF THE LUNG DETERMINED BY HELIUM DILUTION. DESCRIPTION OF THE METHOD AND COMPARISON WITH OTHER PROCEDURES

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INTRODUCTION

The use of the foreign gas, helium, for determination of the pulmonary capacity has been reported previously in preliminary form (1). The present report concerns further experience with this method and comparison of results obtained with results by the Christie method (2) and by the Lassen, Cournand, and Richards modification of the Christie method (3, 4) in normal and emphysematous subjects.

Foreign gas methods for measurement of the residual air or mid-capacity are not new. Van Slyke and Binger (5) employed hydrogen for this purpose. These authors emphasized the danger of explosion with hydrogen-oxygen mixtures and of occasional contamination of hydrogen with arsine. Oxygen dilution methods, especially the method of Christie (2), displaced the foreign gas method. Lassen, Cournand, and Richards (3, 4) noted an "Oxygen Storage Effect" which they believed introduced errors in the determination of pulmonary capacity by Christie's method especially in patients with large mid-capacities. To correct this error, they modified the calculation by Christie's method to allow for observed differences in the concentration of nitrogen between the alveolar air and the spirometer circuit at the end of the rebreathing period. McMichael in England (6) modified the Christie method by matching the oxygen consumption of the subject with a controlled inflow of oxygen so that the total volume of the lung and the spirometer circuit remained constant throughout the rebreathing period. He proved this alteration abolished the "Oxygen Storage Effect."

More recently, McMichael (7) reintroduced the foreign gas principle employing hydrogen and the

warm wire thermal conductivity katharometer for the analysis. He continued the constant volume feature of his earlier method. The use of a foreign gas with a thermal conductivity quite different from that of oxygen and nitrogen greatly facilitated the determination because it eliminated the separate gas analysis at the end of the rebreathing period. The analysis is continuous with the katharometer and the final concentration is read directly from a meter.

Helium, with a thermal conductivity hardly different from that of hydrogen (Table I), eliminates the danger of hydrogen-air mixtures (which are less now than when first emphasized by Binger and Van Slyke) without sacrifice of the merit of McMichael's method. Since publication of our preliminary article, Behnke (8) has mentioned a method employing helium for the determination of the pulmonary capacity by a "washing out" procedure, which is not related to the method here described except in that helium is used. It is more closely related to the "open" method of Cournand and Richards (9, 10).

APPARATUS AND PROCEDURE

The spirometer circuit is diagrammed in Figure 1. The seven-liter cylindrical spirometer with obliterated internal dead space writes the respiratory tracing on a kymograph drum. The volume of the spirometer may be read from the scale and pointer and, by transfer, from the respiratory tracing itself. The subject is connected to the circuit through a rubber mouthpiece on a three-way valve. The expired air passes vertically through a soda-lime canister for most efficient absorption of carbon dioxide. Oxygen may be added at any desired rate through the diaphragm type flow control and meter on a tank of "medical" oxygen. A blower impels

¹ This laboratory was organized under a grant from Commonwealth Fund.

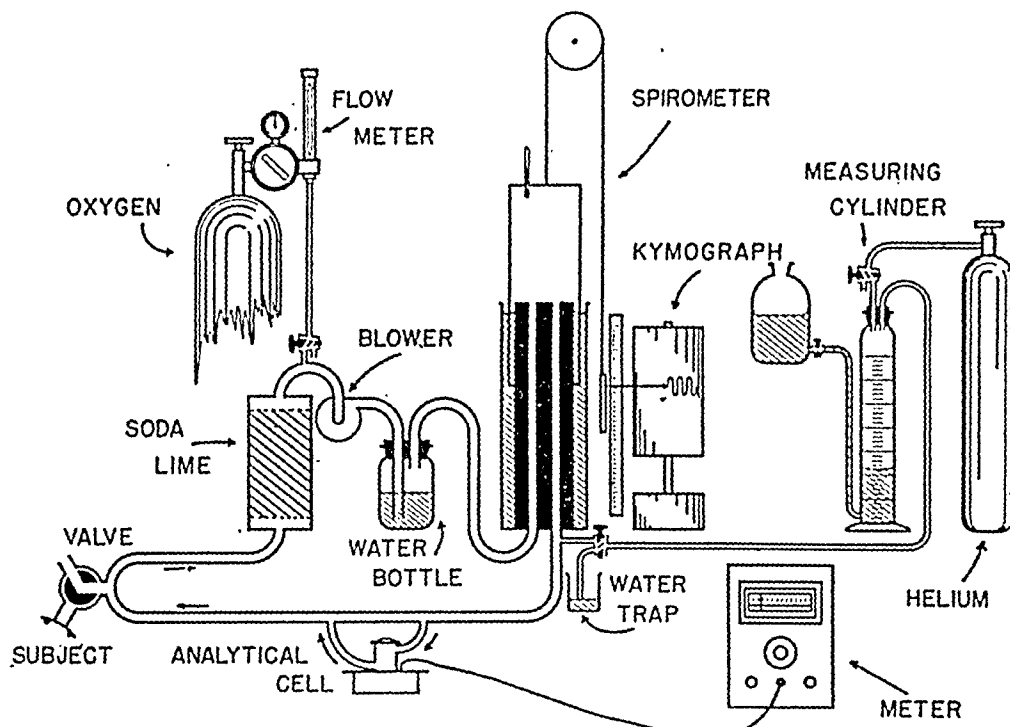


FIG. 1. SPIROMETER CIRCUIT FOR DETERMINATION OF THE VOLUME OF THE LUNG USING HELIUM

he expired air through the water bottle which contains many glass beads to break up the bubbles, desaturate the gas mixture, and prevent disagreeable bumping due to large bubbles. Provision is made for the introduction of helium at the spirometer outlet. Part of the return flow shunts through the analytical cell and returns via the cell outlet to rejoin the return stream. The pressure differences in different segments of the circuit are small: slight negative pressure obtains between the soda-lime and blower, and positive pressure elsewhere. The soda-lime and the water bottle isolate the blower to a degree, preventing vibration of the air column at the mouthpiece.

The dead space of the circuit was determined by rinsing with air, emptying the spirometer, adding a volume of oxygen and mixing with the blower. The final concentration of nitrogen of the dead space air plus added oxygen was determined on the Van Slyke manometric apparatus and the volume corrected for temperature and barometric pressure. Care must be taken to keep the dead space constant by maintaining the water levels in the spirometer and the water bottle.

The oxygen inflow is adjusted to match the oxygen consumption of the subject by taking a short preliminary respiratory tracing, and setting the valve appropriately. This period also ac-

customs the subject to rebreathing. Complementary and reserve volume can be recorded at this time. Any desired volume of helium may be measured in the graduate and delivered into the spirometer by directing the flow with the stopcock at the spirometer base into the spirometer and opening the water reservoir valve. Observations indicated that differences in temperature between the graduated cylinder and the spirometer introduced an error of less than 0.5 ml. in measurement of the helium added. Since this volume was ordinarily 700 ml., this is a negligible error.

The analytical cell² is similar to those used for airplane engine exhaust gas analysis (Figure 2). Complete saturation of the gas mixture is assured by a wet cotton filler in the cell antechamber. When the current through the bridge is maintained constant, and only one component of the gas mixture varies, the indicating meter may be calibrated to indicate the concentration of the gas. For ordinary use, dry cell batteries furnish a suitable cur-

² Cambridge Instrument Co., New York, N. Y. The design of this cell is such that all four chambers are maintained at the same temperature and the chambers containing the standard gas are at atmospheric pressure. The chambers containing the gas to be measured are at the pressure which obtains in the spirometer circuit which is also atmospheric. Gas enters the cells by diffusion, thus eliminating errors due to flow.

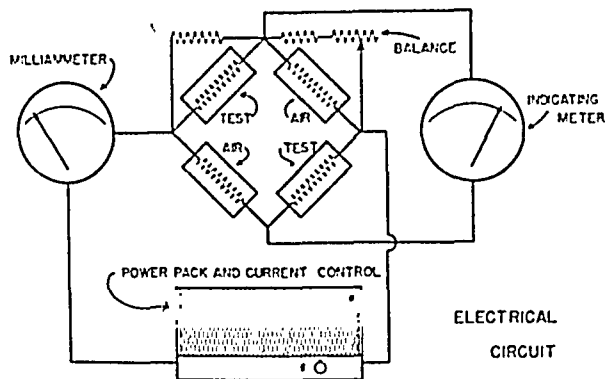


FIG. 2. DIAGRAMMATIC CIRCUIT OF THE KATHAROMETER CELL

rent source. For the extensive studies reported here, the apparatus was in virtually continuous use and a current-controlled power pack was developed which proved more satisfactory. The circuit of the analytical cell and power pack are shown in Figures 2 and 3. With a suitable switching circuit the same meter may readily be used to read the current applied to the bridge as well as the deflections due to imbalance of the bridge.

The analytical cell was calibrated over a range of 0 to 15 per cent of helium by adding measured amounts of helium from the graduated cylinder to measured volumes of air in the spirometer circuit.

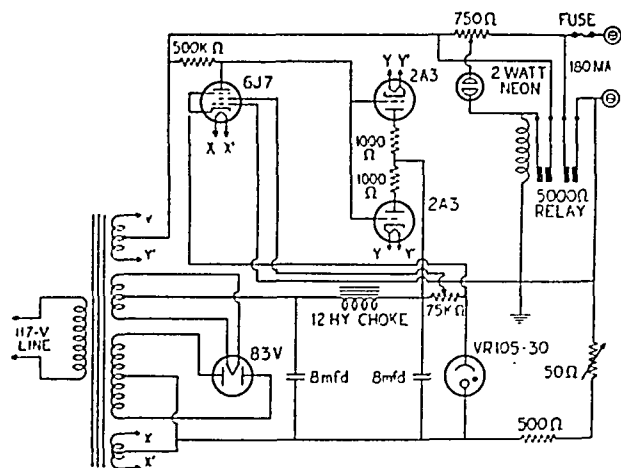


FIG. 3. CIRCUIT OF THE CURRENT CONTROL POWER PACK

Observations during this process, made with the wet and dry bulb thermometers, indicated that unless the water bottle was in the circuit, the saturation of the circulating gas mixtures with water vapor might fall to 70 or even 50 per cent due to the drying action of the soda-lime. The analytical cell is sensitive to variations in water vapor as to any other gas and thus, for calibration and checking at least, the water bottle is necessary. The water bottle also helps prevent heating due to the blower. Calibration by this means is only absolute if the helium is pure, and in this case, 1.8 per cent

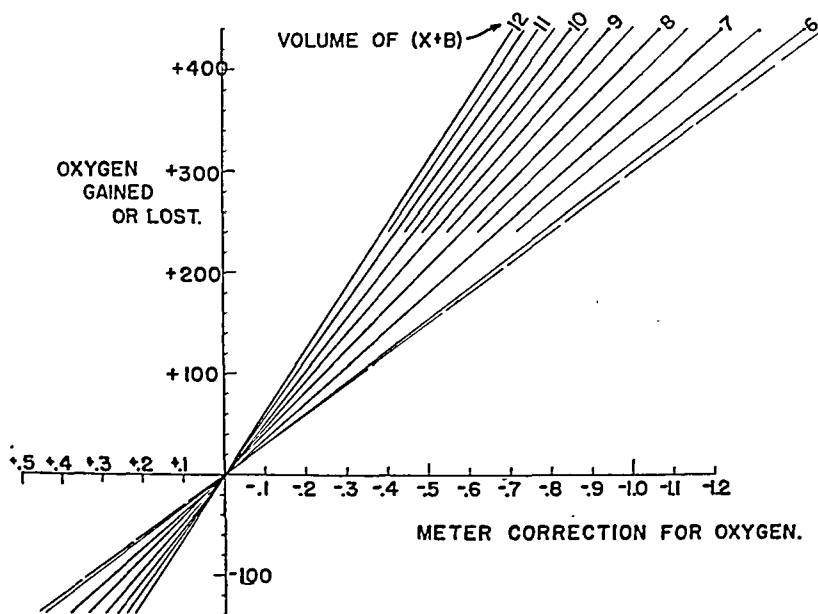


FIG. 4. GRAPH FOR CORRECTION OF METER READING FOR CHANGES IN OXYGEN CONCENTRATION

of "other gases" unspecified were said to be present in the helium.³ There were no means at our disposal to investigate this point more fully, but error is not introduced because the analytical apparatus is used in the same manner as it is calibrated, and the major concern is with observed, not absolute values. Thus 700 ml. from such a supply contains 687.4 ml. of helium.

It proved impossible to match the oxygen consumption of the subject perfectly in every instance and variation in oxygen concentration within the circuit from the beginning to the end of the rebreathing period introduced a small but detectable error, which unfortunately varied depending upon the concentration of helium. Therefore the cell was further calibrated for variation due to oxygen concentration over the range of variation of helium used. This calibration was expressed graphically (Figure 4). A meter correction was obtained by entering this graph with the observed change in oxygen determined from spirometer circuit volume changes and the volume (X + B) obtained as described below.

ANALYTICAL ERROR

The accuracy of the helium analyzer was investigated. This cannot be done by the same means as as that used in calibration because, in this case, there is error in the preparation of the gas mixture due to error in measuring the air in the spirometer and error in the measurement of the helium added to the spirometer as well. Accuracy in calibration depends upon averaging a number of determinations over the whole range. To test the reproducibility of the helium analyzer a tank of

TABLE I

THERMAL CONDUCTIVITY OF SEVERAL GASES
 (CALORIES PER SECOND PER SQUARE CENTIMETER FOR A
 THICKNESS OF ONE CENTIMETER AND A TEMPERATURE
 DIFFERENCE OF 1° CENTIGRADE.)

AIR	5.68 x 10 ⁻⁵
NITROGEN	5.24
OXYGEN	5.63
CARBON DIOXIDE	3.07
HYDROGEN	36.9
HELIUM	33.9

³ Ohio Chemical Company, "Medical helium."

TABLE II

REPEATED ANALYSES ON THE SAME TANK OF A
HELIUM AIR MIXTURE

DATE	HELIUM IN VOLUME %
SEPT. 22	10.13
OCT. 20	10.16
OCT. 22	10.14
OCT. 23	10.18
OCT. 25	10.16
OCT. 27	10.14
OCT. 28	10.30
OCT. 29	10.10
NOV. 1	10.07
NOV. 3	10.10
NOV. 4	10.05
NOV. 5	10.25

MEAN 10.15 VOL. %

STANDARD DEVIATION ±0.07 VOL. %

STANDARD DEVIATION MULTIPLIED BY T FOR N=11 AT THE 95 %
POINT ±0.15 VOL. %.

approximately 10 per cent helium in air was obtained.⁴ This mixture of constant composition was bubbled through water several times and then allowed to flow through the analytical cell which was disconnected from the spirometer circuit for this purpose. The procedure was repeated over a period of several months and the observed concentrations determined with the cell were analyzed statistically (Table II). The mean of these observations which was taken as the "true" concentration of helium in the tank of gas was 10.15 volumes per cent helium with a standard deviation of ±0.071 volumes per cent. Then the analytical "error" in 95 per cent of cases would be less than ±0.15 volumes per cent or 1.5 per cent (11).

This error was compared with the standard error of the analysis for nitrogen in spirometer mixture and alveolar air samples by statistical analysis of 103 serial pairs of duplicate analyses for nitrogen made on the Van Slyke manometric apparatus during this study. One-half of the difference between duplicates was taken as the deviation from "truth." The standard error was ±0.103 volumes per cent. There was no correlation between the magnitude of the error and the concentration of nitrogen. At 80 per cent nitrogen, 95

⁴ Ohio Chemical Company.

per cent of "errors" would lie between ± 0.26 per cent and at 50 per cent nitrogen between ± 0.42 per cent. Thus the analysis for helium by the katharometer carries more error than the analysis for nitrogen in the Van Slyke manometric apparatus, when compared by this method.

MIXING TIME

We investigated the rate at which equilibrium is attained in rebreathing a helium mixture by observing the concentration of helium indicated by the meter at one-minute intervals after turning the subject into the spirometer circuit containing 5,500 ml. of 12.5 per cent helium in air while

keeping the volume constant by adding oxygen at the rate of the subject's oxygen consumption. In these observations, no allowance was made for lag in the meter which does occur because diffusion must take place from the meter antechamber into the cells in the analyzer block. The results of this investigation are shown in Figure 5. At the end of seven minutes equilibrium has been reached in the group with mid-capacities such that lung plus spirometer volume is less than 8 liters. When this volume is larger, there is a notable difference between the six- and the seven-minute concentrations of helium. When the final lung plus spirometer volume equals 9 or 10 liters, the concentration of

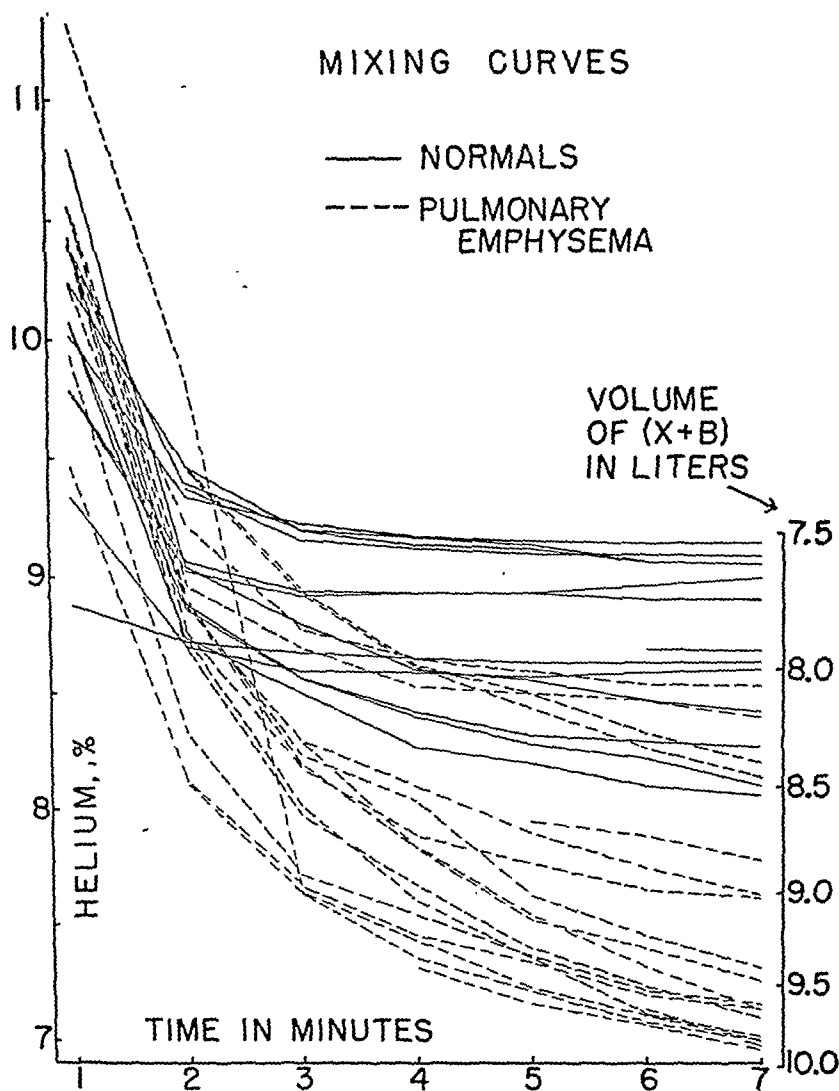


FIG. 5. CONCENTRATION OF HELIUM AT INTERVALS OF ONE MINUTE DURING REBREATHING A 12.5 PER CENT MIXTURE OF HELIUM IN AIR

helium appears still to be falling at a rate of approximately 0.1 per cent of helium per minute. From these observations we might conclude there is an error due to incomplete mixing of helium in the lung in those subjects with large mid-capacities. However, for practical purposes this is not the case. Comparison was made of seven-minute and ten-minute runs in determining the mid-capacity and there is no important or consistent difference (Table III). McMichael (7) read the hydrogen concentration when the "concentration no longer fell" but in some of our normal subjects there was often a small but detectable change in meter deflection, even after 30 minutes of rebreathing. Other factors, especially leaks, begin to be important in prolonged periods of rebreathing. Spirometer circuits are, after all, only relatively gas-tight, and helium has a very marked tendency to escape, due to its low viscosity and other physical properties. Extra precautions against leakage are especially important when using helium. All joints in our

TABLE III

MIDCAPACITY: 7 MINUTE MIXING PERIOD COMPARED WITH 10 MINUTE MIXING PERIOD IN THE SAME SUBJECT.

SUBJECT	DIAGNOSIS	7 MIN.	10 MIN.	10 MIN. - 7 MIN.
W. L.	NORMAL	2.13	2.20	+0.07
J. E.	NORMAL	2.57	2.64	+0.07
J. R.	NORMAL	1.55	1.50	-0.05
A. H.	ARTHRITIC	3.08	3.17	+0.09
C. S.	MILD EMPHYSEMA	1.93	1.98	+0.05
A. D.	MARKED EMPHYSEMA	3.10	3.11	+0.01

equipment were closely fitted and sealed with Glyptal.⁵

The relation of the data presented in Figure 5 to the pulmonary emptying rate data of Cournand, Baldwin, Darling, and Richards (10) is obvious. It should be remembered, however, that the helium meter reading lags behind the actual concentration changes in the spirometer circuit gases and that these in turn are doubtless different from

⁵ General Electric Company.

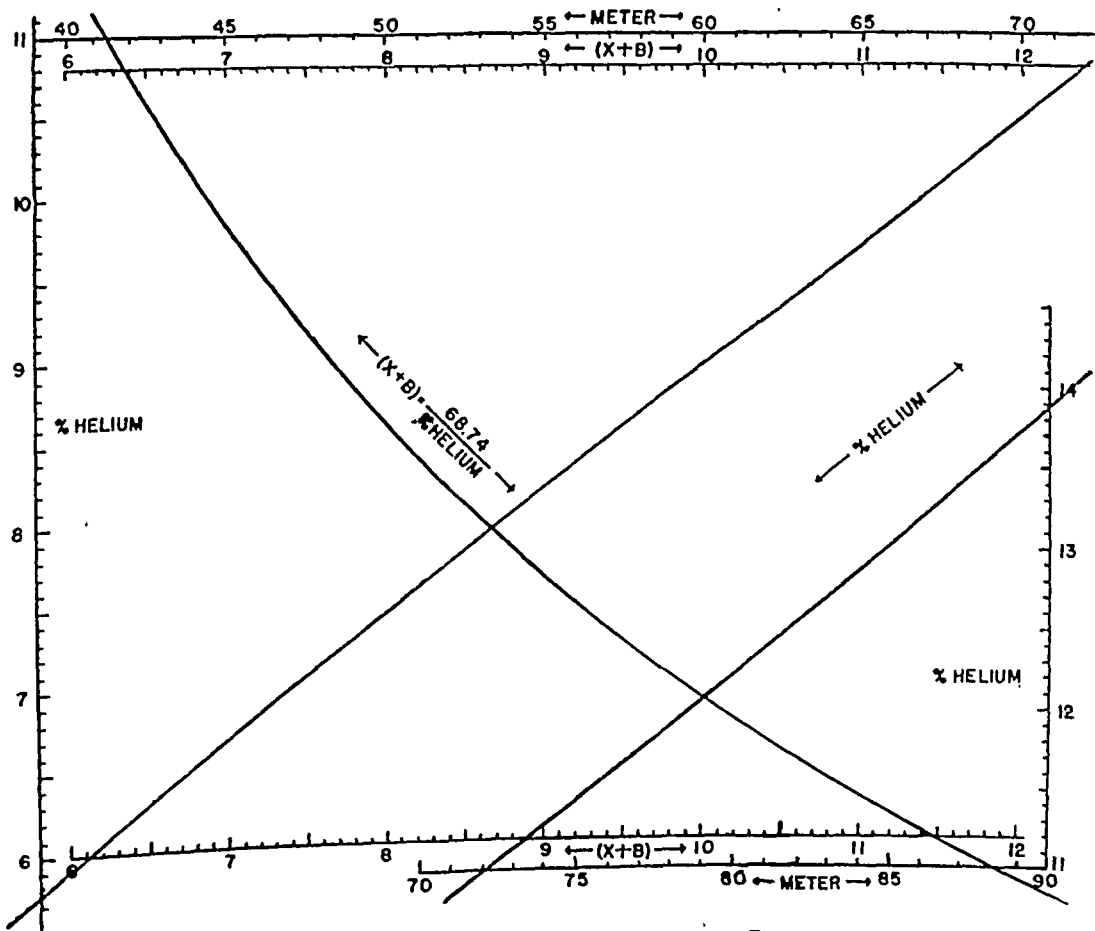


FIG. 6. GRAPH FOR CONVERSION OF METER READING INTO CONCENTRATION OF HELIUM AND TO VOLUME DISTRIBUTION OF HELIUM

alveolar helium concentration changes. Despite this, since conditions affecting this relation are relatively constant, much the same conclusions may be drawn from these curves as from the much more laborious pulmonary emptying rate procedure.

REPRODUCIBILITY

Table IV exhibits 22 pairs of determinations in normal subjects and 13 pairs in emphysematous patients. The difference between duplicates can be used to assess reproducibility of a method (11). The "error" is ± 0.084 liters in normals and ± 0.119 liters in emphysematous patients. When these are corrected for sample size (11) it is found that the "standard error" of a single determination will be less than ± 0.174 liters in normals and less than ± 0.258 liters in emphysematous patients in 95 per cent of cases. Consequently, the error of the mean of a pair of duplicates will be ± 0.123 liters and ± 0.182 liters in normal subjects and emphysematous subjects, respectively, in 95 per cent of cases.

Reproducibility of the method from day to day may be assessed from Table V. The mid-capacity determined in duplicate on different occasions shows deviations of approximately the order of magnitude to be expected from the analysis in the preceding paragraph. "Error" in terms of per

TABLE IV

DUPLICATE LUNG VOLUME DETERMINATIONS WITH HELIUM

FIRST	SECOND	DIFFERENCE	FIRST	SECOND	DIFFERENCE
1.90	1.99	-0.09	2.98	2.97	+0.01
1.70	1.79	-0.09	3.25	3.15	+0.12
1.59	1.40	+0.19	1.82	1.81	+0.01
1.81	1.76	+0.05	2.06	2.13	-0.07
1.56	1.61	-0.05	2.56	2.58	-0.02
1.89	1.74	+0.15	1.62	1.71	-0.09
2.55	2.43	+0.12	3.29	3.31	-0.02
2.28	2.65	-0.37	1.51	1.71	-0.20
2.23	2.15	+0.08	1.91	1.91	0
2.25	2.25	0	1.90	1.85	+0.07
2.16	2.15	+0.01	1.89	1.88	+0.01

STANDARD ERROR ± 0.084

PATIENTS WITH PULMONARY EMPHYSEMA

FIRST	SECOND	DIFFERENCE	FIRST	SECOND	DIFFERENCE
1.93	1.94	-0.01	3.81	3.80	+0.01
3.26	2.94	+0.32	3.89	3.97	-0.08
2.96	2.71	+0.25	4.13	3.98	+0.15
3.04	2.77	+0.27	4.02	3.87	+0.15
3.17	2.90	+0.27	3.66	3.67	-0.01
2.42	2.42	0	3.90	3.97	-0.07
2.51	2.45	+0.06			

STANDARD ERROR ± 0.119

cent for the helium method appears to be less than ± 6 per cent in 95 per cent of cases when the mean of duplicate determinations is used, whether the subjects are normal or emphysematous.

COMPARISON WITH OTHER METHODS: PROCEDURE

The Christie procedure (2) and the modified Christie method of Lassen, Cournand and Richards

TABLE V

REPEATED DETERMINATIONS WITH HELIUM ON DIFFERENT DAYS

	DATE	MID-CAPACITY, LITERS	DEVIATION FROM MEAN
T.A. FEMALE	NOV. 22	1.94	+0.095
NORMAL	DEC. 27	1.75	-0.095
J.A. MALE	DEC. 21	1.50	-0.145
NORMAL	DEC. 28	1.79	+0.145
G.M. MALE	NOV. 8	1.61	-0.10
NORMAL	APRIL 23	1.81	+0.10
E.M. FEMALE	MAY 16	2.46	-0.015
NORMAL	MAY 19	2.49	+0.015
B.M. MALE	SEPT. 1	2.19	-0.01
NORMAL	SEPT. 2	2.25	+0.05
	SEPT. 7	2.16	-0.04
W.L. MALE	DEC. 4	2.06	-0.035
NORMAL	DEC. 8	2.13	+0.035
E.H. MALE	NOV. 18	2.97	-0.11
NORMAL (ARTHRITIC)	DEC. 10	3.19	+0.11
A.B. FEMALE	DEC. 17	3.26	+0.16
MARKED EMPHYSEMA	DEC. 21	2.94	-0.16
W.D. MALE	APRIL 15	2.90	-0.02
EMPHYSEMA	APRIL 16	2.83	-0.09
	APRIL 21	3.03	+0.11

(3, 4) were carried out in the usual way. After these determinations were complete, the subject rested while the oxygen flow valve was adjusted to allow an inflow of oxygen into the spirometer circuit equal to his observed oxygen consumption. Turning off the stopcock between the oxygen tank flow control and the spirometer circuit (see Figure 1) stops the oxygen flow without disturbing the flow rate setting. The spirometer circuit was again well rinsed with room air, and the helium meter turned on. With the current through the bridge set at the standard value, the meter was adjusted to indicate zero with the balancing resistance on the cell (Figure 2). The spirometer circuit was brought to a volume of 4,800 ml. with air, and 700 ml. of helium were added from the graduated cylinder. Two hundred ml. of oxygen were then added to bring the oxygen concentration back to that of room air. As the blower mixed these gases, the helium meter deflected to indicate a concentration of 12.5 per cent helium, confirming the proper functioning of this instrument. Now the subject was allowed to rebreathe for seven minutes. Oxygen flow into the circuit at the previously determined rate was started simultaneously with turning the valve directing the subject into the spirometer circuit. At the end of rebreathing, the subject was turned back to room air and the oxygen flow stopped simultaneously. The subject was disconnected; the temperature was recorded. A few moments were allowed for absorption of carbon dioxide while circulation by the blower continued. Then the final meter reading was recorded. The relation of the initial and final lines on the kymograph tracing to the spirometer scale were recorded. These then constitute a record of the volume changes of the bell. This whole procedure was repeated after a suitable rest period.

Finally, the subject was again connected to the spirometer circuit, and while the kymograph recorded the respiratory tracing, the subject made several maximal expirations with periods of quiet breathing between, then several maximal inspirations. From this tracing the complementary air and reserve air were measured.

Calculations and corrections

The mid-capacity by the original Christie method was calculated from the equation:

$$MC = \frac{N(D + O) - (0.791D + C)}{(0.791 - N)}$$

where MC = mid-capacity.

D = volume of dead space in ml.

O = volume of O₂ in ml. added originally minus O₂ absorbed.

N = per cent concentration of nitrogen in spirometer at end divided by 100.

C = correction for nitrogen in the oxygen added originally for respiratory quotient and for nitrogen excreted from the body during rebreathing.

The modification of Lassen, Cournand and Richards was calculated from this equation:

$$MC = \frac{N(D + O) - (0.791D + C)}{\bar{a} - \bar{p}}$$

where \bar{a} = per cent nitrogen concentration in alveolar air before rebreathing divided by 100.

\bar{p} = per cent nitrogen concentration in alveolar air after rebreathing divided by 100.

Calculation of the mid-capacity by helium required first that the meter reading be corrected for gain or loss of oxygen observed in the respiratory reading (Figure 4). With this corrected value the following equation could be used although graphic methods were actually employed in practice:

$$MC = \frac{QHe}{CHe} - SV$$

where: SV = spirometer circuit volume, i.e., dead space plus spirometer bell volume at expiration.

QHe = ml. helium added before rebreathing.

CHe = final helium per cent concentration divided by 100. This concentration can be obtained from calibration graph entered with the meter reading corrected for changes in O₂ concentration (Figure 6).

Since 0.700 liters of tank gas containing 0.6874 liters of He are always added, the numerator of the above fraction is fixed.

All these mid-capacities must be corrected for temperature, water vapor, and barometric pressure. For this the customary equation was used:

$$MC \text{ cor.} = MC \text{ obs.} \times \frac{(760 - P_t)}{(760 - 45)} \times \frac{(273 - 37)}{(273 - T)}$$

where MC cor. = mid-capacity corrected for temperature and barometric pressure.

MC obs. = mid-capacity observed.

T = final spirometer temperature in degrees C.

P_t = partial pressure of water vapor in mm. of Hg for T.

45 = partial pressure of water vapor at body temperature 37° C.

In actual practice graphic methods as introduced by McMichael (6) were used. Figure 6 is a composite graph of the relation between the meter reading, the concentration of helium, and the volume in which 0.6874 liters of helium must be distributed if the concentration is as indicated. The upper horizontal line of the graph was entered with the meter reading and a perpendicular dropped from this reading to the helium concentration line. From this

point, by reading horizontally to the helium scale, the concentration of helium could be obtained. At the point where this horizontal intercepted the curved line denoting the volume of distribution of helium, one may read vertically to the horizontal scale of values for $(X + B)$, which represents the total volume of lung and spirometer system. However, this reading is in error due to variation in oxygen concentration. The observations of variation of meter reading for variation in terms of oxygen gained or lost for various values of $(X + B)$: that is, at various concentrations of helium (Figure 4). The tentative value of $(X + B)$ together with the oxygen gained or lost was used to enter Figure 4 to obtain a meter correction for oxygen. With this new corrected meter reading, the composite graph (Figure 6) was again entered, reading vertically from the meter scale to the helium concentration line, then horizontally to the volume of $(X + B)$ curve, thence vertically to the scale of $(X + B)$. $(X + B)$ corresponds to $\frac{Q_{He}}{C_{He}}$, X corresponds to mid-capacity and B corresponds to SV, the spirometer circuit volume.

The helium mid-capacity requires further correction. The dead space of the mouthpiece and valve was 30 ml. Assuming a respiratory quotient of average value 0.8 and 2 liters for the mid-capacity, there is an apparent increase of 30 ml. in the mid-capacity due to differences in concentration of inert gases between the lung and the spirometer system. In our earlier report (1) we stated that 10 ml. of helium were absorbed by the body during the period of rebreathing. Extension of the experiments by which we arrived at this figure cast doubt upon their validity. Ten ml. of helium distributed in a volume of approximately 8 liters of lung plus spirometer circuit, is not easy to find by analysis for differences in concentration, for at a helium concentration of around 12.5 per cent, in this 8-liter volume, a 10-ml. change in quantity of helium would result in a change of 12.5 volumes per cent or 1.56 per cent in the concentration. Since the error of analysis is of the same magnitude, determination of changes in concentration of helium after equilibrium between lung and spirometer has been reached cannot be expected to show this small magnitude reliably. As previously mentioned, methods of accurate absolute analysis for helium in the presence of other gases were not available. Proper investigation of this point remains for the future. Some helium must inevitably be absorbed during rebreathing, and if it were much more than 10 ml., we could have detected it with some accuracy. Therefore we have continued to subtract 110 ml. from the observed mid-capacity which is the amount attributable to a loss of 0 ml. of helium with an average mid-capacity of 2 liters. It is then to be remembered that all the mid-capacities by helium are made 110 ml. smaller by this rather arbitrary correction. In practice, 180 ml. were subtracted as the rounded total of the three corrections: mouthpiece, R.Q., and helium absorption. The mouthpiece volume correction is applied to the other calculations also, and the difference in inert gas between lung and spirometer due to R.Q. is also written into the equation $(Q.V.)$.

RESULTS OF COMPARISON WITH OTHER METHODS

The original Christie (2) mid-capacity method and the Lassen, Comar and Richards (3, 4) modification of the original Christie method are compared with the new helium method in ten normal subjects and eight patients with pronounced pulmonary emphysema in Table VI. Each determination was done in duplicate. There is less variation between duplicates by the helium method than in either of the other two methods, not only in normals but also in patients with emphysema. From the last two lines of Table VI it is seen that in the normal the volume determined with helium is virtually always smaller than the volume determined by the other two methods by an amount of approximately 200 ml. and 100 ml., respectively.

In this connection it should be recalled that the rather arbitrary correction for helium taken up by the body is of this same order of magnitude. We have no objective proof of the absorption of helium, although we know from purely physical considerations that some must take place.

It may be observed that differences between the original Christie and the corrected Christie, while fairly consistent in the normal subjects, are not at all so in those patients with emphysema, although according to the "oxygen storage" concept the difference should be more marked in this group. The original method gives values in normals consistently higher by about 1/10 liter. However, in the emphysematous patients no such consistency is observed and indeed the mean difference is in the opposite direction. The explanation of this inconsistency in the emphysematous patients is found in the variable relation of their initial and final alveolar air samples to outside air and to final spirometer nitrogen, respectively. It was our opinion that the corrected mid-capacity did not offer any advantage over the original method in pathological subjects despite the theoretical advantages described for it. In a personal communication the authors of the corrected method informed us that they now prefer their open method (9).

CONCLUSIONS

The principal advantage of this method of determining mid-capacity is the speed with which determinations may be carried out, together with evidence indicating a higher degree of reproducibility.

Figure 1

TABLE VI The Lassen, Courmand and Richards (3, 4) modification of the original Christie method in ten normal and eight emphysematous subjects																		
Comparison of the results obtained with original Christie method (2), the Lassen, Courmand and Richards (3, 4) modification, and the new helium method in ten normal and eight emphysematous subjects																		
Subject	Normal										Emphysematous subjects							
	T. A.	J. A.	C. M.	D. E.	R. W.	J. J.	L. S.	J. F.	E. F.	E. M.	W. D.	W. M.	E. W.	R. D.	F. P.	F. E.	S. R.	G. P.
Sex	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
Age	26	26	29	25	22	32	16	30	25	28	61	76	63	51	24	46	55	55
Height, cm.	176.5	176.5	190.0	175.5	185.5	170.0	164.0	173.0	161.0	169.5	158.0	161.5	165.0	168.5	153.0	162.0	171.0	175.0
Weight, kg.	62.0	70.5	114.0	72.0	79.5	68.0	62.4	70.2	57.0	63.0	58.0	53.6	54.7	55.4	51.8	51.0	64.0	66.0
First run spirometer N ₂ % minus spiro N ₂ %	52.8	50.2	50.5	52.0	59.7	49.1	51.1	51.1	45.5	51.7	55.8	52.5	51.1	54.1	52.3	47.5	53.3	51.5
First final alveolar air N ₂ % minus spiro N ₂ %	47.1	40.5	+1.3	+1.3	59.7	+1.3	+0.3	+0.7	+1.1	+0.3	+2.6	+2.2	+2.1	+2.5	+3.0	+1.1	+2.3	+3.1
First final alveolar air N ₂ % minus spiro N ₂ %	+0.3	-0.1	50.9	+1.6	+1.0	-0.3	51.0	49.7	46.1	+0.3	51.5	53.7	52.6	49.5	52.6	47.6	55.9	52.6
Second run spirometer N ₂ % minus spiro N ₂ %	+0.2	50.2	51.2	+1.0	58.8	48.6	51.0	49.7	46.1	+0.3	51.5	53.7	52.6	49.5	52.6	47.6	55.9	52.6
Second final alveolar air N ₂ % minus spiro N ₂ %	46.7	+0.1	51.2	+1.0	58.8	48.6	51.0	49.7	46.1	+0.3	51.5	53.7	52.6	49.5	52.6	47.6	55.9	52.6
Second initial alveolar air N ₂ % minus spiro N ₂ %	+0.1	1.84	+0.2	1.92	1.87	3.87	1.94	2.93	2.03	2.03	2.41	2.55	2.18	3.76	3.66	2.56	3.07	3.93
Original Christie mid-capacity, liters	0	1.96	-0.06	1.84	1.86	3.61	1.84	2.93	2.03	2.03	2.41	2.55	2.18	3.76	3.66	2.56	3.07	3.93
Second Christie mid-capacity, liters	-0.01	-0.09	1.79	1.81	1.67	3.30	1.61	2.93	2.03	2.03	2.41	2.55	2.18	3.76	3.66	2.56	3.07	3.93
Original Christie mid-capacity, first and second runs	1.75	+0.05	4.04	4.86	3.74	4.29	3.36	2.78	3.40	2.94	2.72	2.36	2.18	3.76	3.66	2.56	3.07	3.93
Difference between first and second runs	-0.09	0.31	0.64	0.56	0.68	0.68	0.96	0.74	0.74	0.58	0.58	0.56	0.56	0.57	0.57	0.56	0.57	0.57
Corrected difference between first and second runs	2.82	2.20	4.68	4.86	3.74	4.29	3.36	2.78	3.40	2.94	2.72	2.36	2.18	3.76	3.66	2.56	3.07	3.93
Helium mid-capacity, first and second runs	+0.13	+0.06	+0.05	+0.11	+0.20	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Helium mid-capacity, first and second runs	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Reserve air, liters	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Complemental air, liters	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Vital capacity, liters to total capacity, helium	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Vital capacity, liters to total capacity, nitrogen	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Ratio of residual air to total capacity, helium	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Ratio of residual air to total capacity, nitrogen	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Mid-capacity Christie minus mid-capacity helium	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Mid-capacity Christie minus mid-capacity nitrogen	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Mid-capacity corrected minus mid-capacity helium	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09
Mid-capacity corrected minus mid-capacity nitrogen	+0.31	+0.27	+0.01	+0.03	+0.19	+0.57	+0.10	+0.17	+0.09	+0.15	+0.16	+0.12	+0.13	+0.13	+0.11	+0.10	+0.09	+0.09

The method has been employed in the Cardio-Respiratory Laboratory of the University of Rochester for several years with very satisfactory results. The determination of mid-capacity is growing in clinical recognition, particularly in relation to the diagnosis of pulmonary emphysema. The facility with which the determination can be made will make possible broader application of this valuable test. It is evident that a number of steps might well be taken to simplify and further improve the method described. Particular attention ought to be devoted to reduction of spirometer circuit volume and increases in accuracy might be secured by employing higher concentrations of helium.

SUMMARY

1. A full description of a new method for the determination of the volume of the lung has been presented.
2. Graphic methods were employed for rapid solution of the calculations involved.
3. Data are presented to permit critical evaluation of the method as to reproducibility and as to comparison with two other methods.
4. The rapidity and accuracy of this new method recommend it for clinical application.

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STUDIES ON HUMAN ALCAPTONURIA. EFFECT OF THIOURACIL, PARA-AMINOBENZOIC ACID AND DI-IODOTYROSINE ON EXCRETION OF HOMOGENTISIC ACID

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The literature on alcaptonuria has been well summarized (1-6).

Paschke *et al.* (7) reported that thiouracil and para-aminobenzoic acid inhibited the *in vitro* conversion of tyrosine to melanin by tyrosinase, and White (8) found that thiouracil inhibited melanuria. Lerner and associates (9) noted that the tyrosinase of mouse melanoma is a copper protein whose activity can be inhibited by the addition of substances which combine with copper (phenylthiourea and others) and then restored by the addition of an excess of copper. Since homogentisic acid is an oxidative product of tyrosine, it seemed worthwhile to determine whether the production of homogentisic acid could be inhibited by thiouracil and para-aminobenzoic acid. When 3, 5, di-iodotyrosine is fed to rabbits, about 60% (Foster and Gutman [10]) is excreted as 3, 5, di-iodo-4-hydroxyphenyllactic acid. It, therefore, occurred to us that, since the number 5 carbon on the benzene ring is blocked by iodine, the alcaptonuric patient would not be able to utilize di-iodotyrosine as he would l-tyrosine to form homogentisic acid (2, 5, dihydroxyphenylacetic acid).³

Soderbergh (12) observed that the daily administration of 3.0 gm. of potassium iodide to an alcaptonuric decreased the urinary excretion of homogentisic acid by 2.0 gm. per day. This compound was administered to our patient in an attempt to influence similarly the excretion of homogentisic acid.

EXPERIMENTAL

The subject of our study was a 59-year-old white male, M.N., History No. 43082, who noted a blackish discolora-

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³ After this experiment had been completed, we learned that in 1907 Abderhalden, Bloch, and Rona (11) fed 2 gm. of 3, 5, di-iodotyrosine to an alcaptonuric without increasing the production of homogentisic acid.

tion of the ears 25 years ago, at which time it was observed that his urine became black on standing. Stiffness of almost all the joints occurred five years later.

Extensive laboratory examinations were essentially normal except for a blood uric acid of 6.8 mgm.% and the presence of homogentisic acid in the urine. The serological reactions for syphilis were negative.

Roentgenologic examinations were reported as follows. *Spine*: Marked hypertrophic changes were present. The intervertebral discs showed calcification, especially in the lumbar region. *Pelvis*: The right hip space was considerably narrowed and there was eburnation of the contiguous portions of the joint. *Knees*: There was calcification of the quadriceps tendons bilaterally. Hypertrophic changes were seen in both knee joints. *Legs*: Linear, irregular, and amorphous calcifications were seen in the soft tissues in the lower half of both legs. There was a mild osteoporosis of all bones. The ankles and feet showed no pathological changes. The changes described were considered characteristic of alcaptonuric ochronosis.

The genetic history of this patient is of interest. His parents were first cousins, neither of whom had alcaptonuria. The patient married a cousin who has no alcaptonuria. The presence of alcaptonuria and arthritis is indicated in Figure 1.

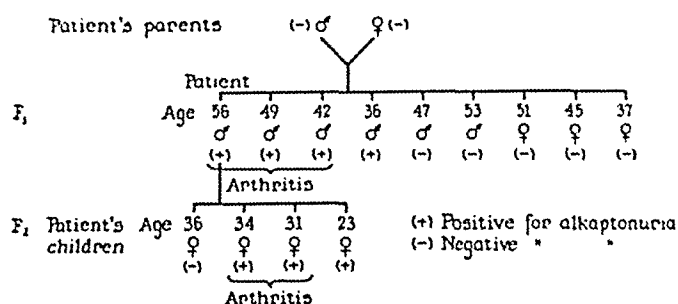


FIG. 1. GENETIC HISTORY OF ALCAPTONURIC PATIENT WHO WAS SUBJECT OF PRESENT STUDY

The effect of thiouracil and para-aminobenzoic acid on the urinary excretion of homogentisic acid was determined. In addition, the effects of potassium iodide, di-iodotyrosine and l-tyrosine were also observed.

Period 1: Control period of seven days. *Period 2:* Thiouracil 0.2 gm. t.i.d. was administered for 12 days. *Period 3:* Para-aminobenzoic acid was then given in doses of 4 gm., five times a day, with 30 c.c. of 5% NaHCO₃ accompanying each dose, for seven days. *Pe-*

TABLE I

Period	Day of experiment	Urine volume c.c./24 hours	Homogentisic acid gm./24 hours
I Control	1	1695	4.41
	2	1560	4.84
	3	1240	3.60
	4	2580	4.89
	5	1690	4.56
	6	2330	6.76
	7	1650	3.80
	Daily average for period 4.69		
II Thiouracil 0.2[gm., t.i.d.	8	1320)*	4.88)*
	9	1320}	4.88}
	10	1810	5.07
	11	2380	5.48
	12	1580	4.74
	13	1750)*	4.16)*
	14	1750}	4.16}
	15	1850	4.44
	16	1120	3.70
	17	1950	5.27
	18	1380	3.31
	19	1180	4.25
	Daily average for period 4.53		
III Para-aminobenzoic acid 20 gm./day	20	1840	4.23
	21	1850	4.81
	22	1630	4.08
	23	1995	4.99
	24	1735	4.86
	25	1950	5.46
	26	1325	4.24
	Daily average for period 4.67		
IV Control	27	1140	4.33
	28	1460	4.09
	29	1510	3.62
	30	1290	4.26
	31	1090	3.14
	Daily average for period 4.09		
V Potassium iodide 2.0 gm./day	32	1860	5.21
	33	1040	3.64
	34	2030	4.87
	Daily average for period 4.57		
VI Potassium iodide 4.0 gm./day	35	1700	3.91
	36	1715	3.60
	37	1580	4.42
	38	1460	3.50
	Daily average for period 3.86		
VII 3,5-di-iodotyrosine 8.0 gm./day	39	1270	3.94
	40	1480	4.44
	41	1385	5.40
	42	1480	6.36
	43	1310	4.45
	Daily average for period 4.92		
VIII l-tyrosine 8.0 gm./day	44	1610	4.99
	45	1380	7.87
	46	1410	8.88
	47	1810	8.69
	48	1720	9.46
	Daily average for period 7.98		

* 48-hour urine collection and homogentisic acid output expressed per 24 hours.

riod 4: Control period of five days. *Period 5:* 2.0 gm. of potassium iodide per day were given orally as a 50% solution for three days. *Period 6:* 4.0 gm. of potassium iodide per day were given orally as a 50% solution for four days. *Period 7:* 8.0 gm. daily of di-iodotyrosine were given orally as a powder in four divided doses of 2.0 gm. each for five days. *Period 8:* 8.0 gm. daily of l-tyrosine were given orally as a powder in four divided doses of 2.0 gm. each for five days.

The daily diet during the entire period contained approximately 70 gm. protein and 1800 calories.

METHODS

Twenty-four-hour urine specimens were collected and preserved with approximately 10 c.c. concentrated HCl so that all specimens remained acid to congo red.

Homogentisic acid was determined quantitatively by the iodometric method as described by Lieb and Lanyar (13).

RESULTS AND DISCUSSION

Results are presented in Table I and Figure 2.

The average daily excretions of homogentisic acid in the control periods, 4.69 and 4.09 gm., agree well with the value of 4.65 gm. observed by Neuberger (5) in a patient receiving 60 gm. of protein daily.

Thiouracil and para-aminobenzoic acid did not inhibit the excretion of homogentisic acid since the average daily excretion was 4.53 gm. and 4.67 gm. for the respective periods. It, therefore, seems that the oxidative enzymes involved in the conversion of tyrosine to homogentisic acid are not similar to the tyrosinase concerned with the oxidation of tyrosine to melanin.

When 8.0 gm. of l-tyrosine were administered, the average daily excretion for this period was 7.98 gm. as compared with 4.09 gm. for the preceding control period. The difference, 3.89 gm., represents a conversion of 52.6%. Mittelbach (14) observed a low percentage of conversion to homogentisic acid when 15.0 gm. of l-tyrosine were given. This is in contrast with the 80% to 85% conversion of 3.0 gm. of l-phenylalanine observed by Neuberger (5).

Despite the large amounts (8.0 gm. daily) of 3, 5, di-iodotyrosine administered to our patient, the average daily excretion of homogentisic acid was 4.92, and this did not differ markedly from the control excretions. This would indicate an inability of the alcaptonuric to convert this compound to homogentisic acid.

Foster and Gutman (10) found that when 3, 5, di-iodotyrosine was fed to rabbits, about 60% was

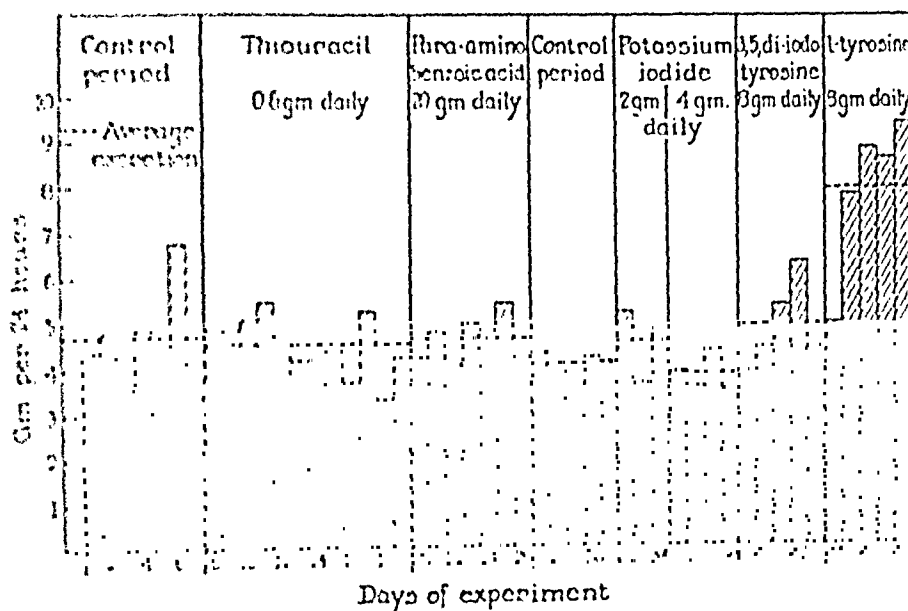


FIG. 2. THE EXCRETION OF HOMOGENTISIC ACID IN GRAMS PER 24-HOUR PERIODS UNDER VARIOUS EXPERIMENTAL CONDITIONS

The amount of homogentisic acid is represented to the nearest tenth of a gram.

excreted as 3, 5, di-iodo-4-hydroxyphenyllactic acid. We did not expect this portion to be converted to homogentisic acid by our patient because the number 5 carbon was blocked by iodine. However, the remaining 40%, representing 3.2 gm. of the daily 8.0 gm. dose administered, might be available for conversion to homogentisic acid. This did not happen.

Soderbergh (12) observed that when an alcaptonuric patient was given 3.0 gm. of potassium iodide daily, the urinary output of homogentisic acid decreased to 6.50 gm. daily from a control level of 8.50 gm. We did not find any decrease in our patient.

CONCLUSIONS

1. An alcaptonuric patient on an approximately 70 gm. daily protein basal diet excreted a daily average of 4.69 gm. and 4.09 gm. of homogentisic acid during two control periods of seven days and five days respectively.

2. Thiouracil and para-aminobenzoic acid did not reduce the excretion of homogentisic acid.

3. 8.0 gm. daily of L-tyrosine yielded an average daily excretion of 7.98 gm. of homogentisic acid, which represented a conversion of 52.6%.

4. 8.0 gm. daily of 3, 5, di-iodotyrosine resulted in an average daily excretion of 4.92 gm. of homogentisic acid, essentially the control level.

5. Potassium iodide in doses of 2.0 gm. and 4.0 gm. daily did not decrease the urinary excretion of homogentisic acid.

6. The oxidative enzymes involved in the conversion of tyrosine to homogentisic acid differ in their lack of response to thiouracil from the tyrosinase which produces melanin.

ACKNOWLEDGMENT

We wish to extend our thanks for the advice and encouragement given by Dr. Louis Leiter, Chief of the Medical Division, Montefiore Hospital.

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THE SIGNIFICANCE OF THE DIFFERENCE IN SYSTEMIC ARTERIAL AND VENOUS PLASMA CONCENTRATIONS IN RENAL CLEARANCE METHODS

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Diodrast clearance values reported in the literature have been calculated partly on constant and partly on rising or falling plasma concentrations. However, we have observed unexpectedly low clearance values on rapidly falling concentration, and as far as we know this phenomenon has not been reported before.

Published values of the diodrast clearance in normal subjects differ with the different techniques applied, and these differences may be explained in part, on the basis of our observations. By analyzing White, Findley and Edwards' (1) results we see that in the subjects where continuous infusion was applied, the average diodrast clearance was 517 ml./min., while in the subjects where falling plasma concentration after a single injection was used, the value was 410 ml./min. One of us, (Hilden [2]), by application of an intravenous single injection, obtained an average clearance of 411 ml./min., whereas by subcutaneous injection, which gives a far more slowly falling plasma concentration, obtained a clearance of 613 ml./min. Goldring, Chasis, Ranges and Smith (3), who used continuous infusion, indicate the normal value to be 688 ml./min. Josephson (4) recently stated that he finds lower diodrast clearance values on the basis of spontaneously falling plasma concentrations than after a single intramuscular diodrast injection.

A few instances will be given first of the above phenomenon. Figure 1a shows the diodrast clearance at different plasma concentrations estimated on rising and falling concentration. Values calculated on falling plasma concentration at about 10 mgm. per cent cease to increase and in fact begin to decline. In comparison it may be seen that the values calculated on rising concentration at the same low level range from about 700 to about 1000 ml./min. The question of why the values vary so much during rising concentration will be discussed later. Similar results are plotted in

Figure 1b. From the moment the concentration begins to fall spontaneously the clearance declines at an increasing rate the lower the concentration. Here, too, the values are essentially higher when calculated on rising concentration. In Figure 1c the diodrast clearance values calculated on a falling curve have been compared with the diodrast clearance estimated on constant blood concentration; a marked difference is noted again. Figure 1d illustrates an experiment, in which a spontaneous fall in the plasma concentration was elicited over one hour and a half, and when the plasma concentration had fallen to 8 mgm. per cent, diodrast was infused intravenously, and the plasma concentration increased. The diodrast clearance increased from about 400 ml./min. to about 600 ml./min. at the same plasma concentration. Figure 1e shows an experiment beginning with an intravenous infusion of diodrast, which brought about a rapid rise in the plasma concentration. Then the infusion was discontinued with the result that the plasma concentration began to fall spontaneously. The diodrast clearances calculated on the rising curve were found to exceed those on the falling curve. The turn occurred at the moment the infusion was discontinued.

A total of 16 experiments were performed. Low clearance values on falling plasma concentration were unquestionable in 12 and in the remaining four the phenomenon was questionable or not present. The reason why the phenomenon may occasionally fail to occur will be mentioned later.

We shall now discuss the possibilities which we have considered in trying to explain the phenomenon.

a. In the above experiments *no* correction was made for delay time; by delay time we mean the time it takes for the urine to pass through the kidneys and the urinary tract from the moment of production until it has reached the bladder. The urine discharged from the bladder at a given point

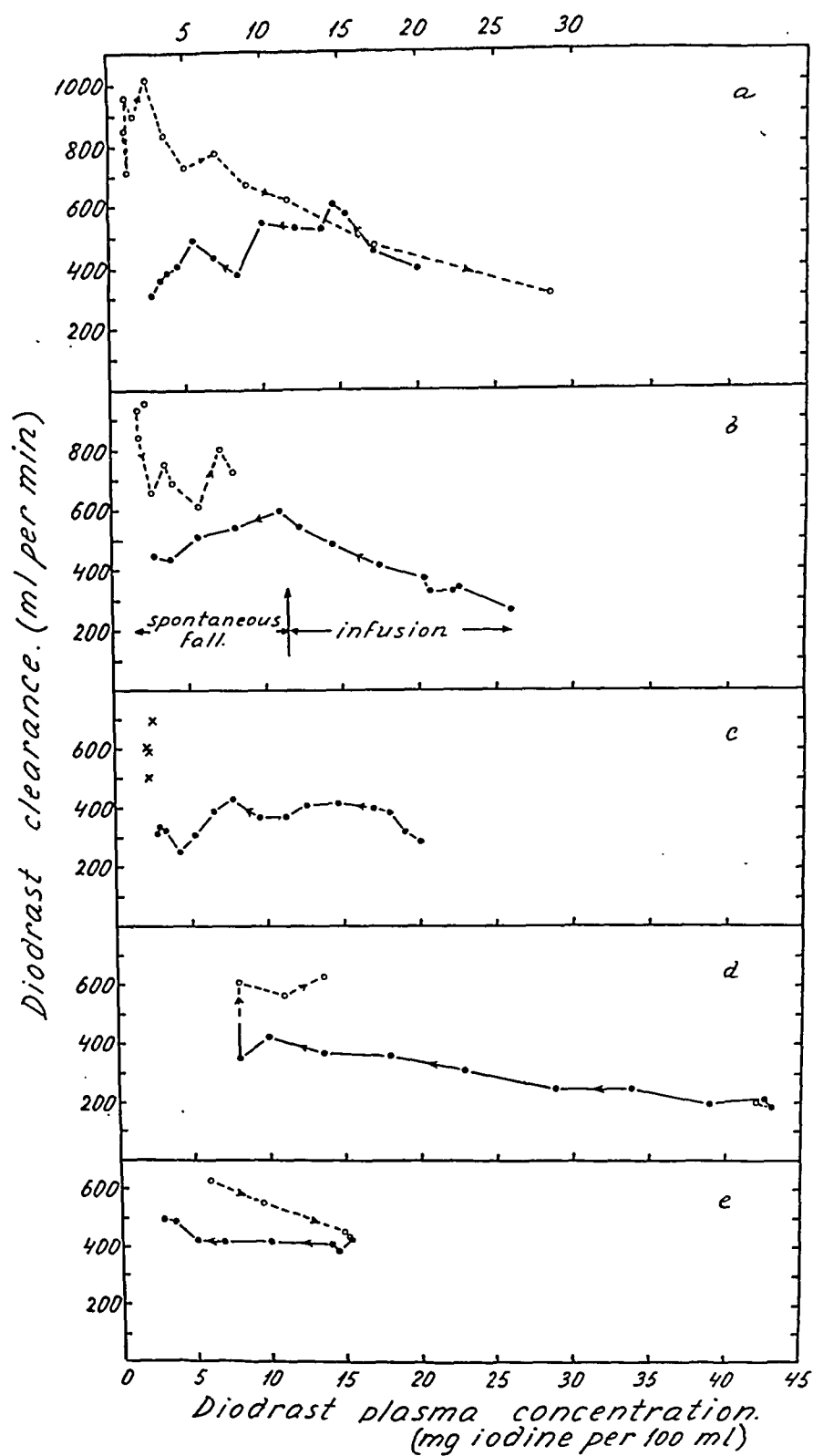


FIG. 1 (a through e). DIODRAST CLEARANCE VALUES CALCULATED ON FALLING AND RISING PLASMA CONCENTRATIONS

Solid dots represent falling, and open circles rising concentrations.

of time is thus produced in the kidneys some five to 15 minutes previously (depending on, among other things, the urine volume). When working with rapidly varying plasma concentrations there may, accordingly, be a great difference between the concentration during which the urine is actually produced and that used in the clearance period. Clearance values determined on falling plasma concentration therefore will be calculated too high. Consequently, omitting correction for delay time cannot alone explain our observations.

b. If diodrast is deposited in the renal tissue for some time before being excreted the diodrast clearance will likewise be calculated too high, because at the high concentrations the excretion will lag somewhat behind the plasma curve.

c. Furthermore, the possibility might be conceived that, at rapidly falling plasma concentration, the erythrocytes were not able to give off their contents of diodrast during the rapid passage through the kidneys. This would result in a shift in favor of the erythrocytes of the normal equilibrium between the diodrast concentrations in blood cells and plasma. The erythrocytes might be conceived to give off diodrast to the plasma after the blood sample had been taken, and the diodrast concentration in plasma accordingly be analyzed as higher than it had been when the blood passed through the kidneys. To elucidate this fact, we compared the diodrast concentrations in blood plasma centrifuged off immediately after withdrawal of blood, and plasma which had been left standing with erythrocytes for an hour after withdrawal. This analysis showed that no diodrast is given off from the blood cells, and that accordingly no such process can explain the phenomenon.

d. Differences in the binding of diodrast to the plasma protein substances, at falling and rising plasma concentration, might likewise be conceived to be responsible for a difference in the plasma excretion of diodrast in the kidneys. This seems rather unlikely, however, since the dissociation of the diodrast-protein complex is known to take place very quickly; the blood is, at least at plasma concentrations below that at which the clearance is self-depressed, almost completely cleared of diodrast by a single passage through the kidneys.

Thus, none of the four possibilities mentioned above appear to explain the low diodrast clearance

values calculated on falling plasma concentrations. However, we believe we have found an explanation in another possibility; the diodrast concentration in arterial blood may differ considerably from that in venous blood, when the plasma concentration is changing quickly.

e. The distribution of diodrast in the circulation, *while the concentration falls*, must be supposed to occur in the following way: the renal venous blood, which is almost or completely free of diodrast, is led to the right half of the heart, where it is mixed with diodrast-containing venous blood from the extrarenal part of the organism. The concentration in the mixture will be identical with that in the arterial blood. It appears, therefore, that the diodrast concentration in arterial blood must be lower than that in the venous blood from the arm generally applied for analysis. Since the clearance (UV/P) ought to be calculated on the basis of the concentration of arterial blood from which diodrast is actually excreted by the kidneys, then the clearance values will be calculated too low when the venous blood concentration is used in the formula.

To this hypothesis the objection might be raised that the above difference between the concentrations in arterial and venous blood always must be present, since the renal venous blood in normal individuals always must represent an afflux poor in diodrast to the heart. If one, however, maintains a *constant* diodrast plasma concentration by continuous infusion, the concentrations in arterial and in mixed venous blood will be identical; the concentration is maintained by supplying diodrast to the venous blood at a rate equal to that at which it is being excreted through the kidneys.

During rising plasma concentration the arterial blood concentration should accordingly be higher than the peripheral venous blood, because the mixed venous blood is being fortified at a rate greater than the rate of excretion. The mixed venous blood (and the arterial blood) will have, therefore, a higher concentration than peripheral venous blood. The arteriovenous difference will depend mainly on the relative rates of loss and infusion.

On the basis of these considerations we have determined the relative concentrations in arterial and venous blood under conditions of rising, falling and constant diodrast concentration.

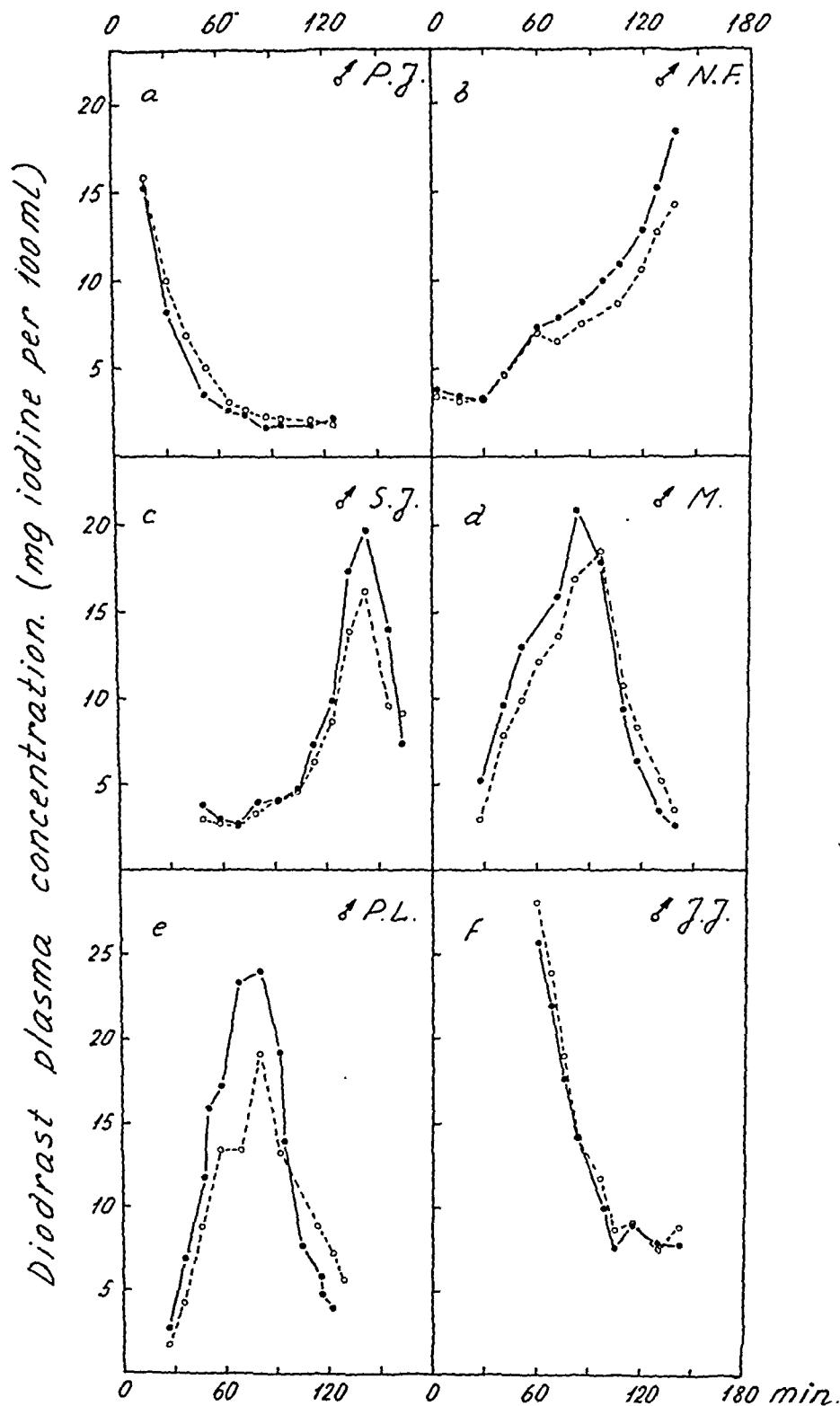


FIG. 2 (a through f). DIODRAST PLASMA CONCENTRATIONS IN ARTERIAL AND VENOUS BLOOD ON RISING AND FALLING PLASMA CONCENTRATIONS

Solid dots represent concentrations in arterial, and open circles concentrations in venous blood.

TABLE 1
Experimental results from subjects P. L. and M. (male)

Di. Diet															
Inulin				Clearance*				Plasma				Urine	Clearance*		
Urine collection period	Urine volume +100 ml. water	Urine volume ml./min.	Delay time min.	Plasma		Urine mgm./100 ml.	Clearance		mgm. iodine/100 ml.	mgm. iodine/100 ml.	mgm. iodine/100 ml.	mgm. iodine/100 ml.	ml./min.	ml./min.	ml./min.
				Arterial	Venous		Arterial	Venous							
min.	ml.	ml./min.	min.	mgm./100 ml.	mgm./100 ml.	mgm./100 ml.	ml./min.	ml./min.	ml./min.	ml./min.	ml./min.	ml./min.	ml./min.	ml./min.	ml./min.
9:50	115	1.5	8.0	16.6	14.4	132	93	107	5.7	3.5	2.5	233	478	779	1035
12:40	122	1.7	8.0	30.0	25.0	282	91	109	13.0	8.1	8.0	434	322	517	522
9:40	124	2.5	7.0	38.8	33.4	335	111	129	17.2	12.0	16.2	441	329	472	348
10:10	139	3.8	6.0	45.8	36.4	305	91	115	21.6	14.8	19.1	366	232	338	262
12:10	162	5.1	5.0	47.2	38.8	420	119	144	23.5	17.8	23.2	447	253	334	257
13:00	153	4.1	6.0	38.0	38.4	373	116	114	19.0	14.5	21.0	425	263	344	238
10:20	137	3.6	6.0	28.4	30.8	272	127	117	8.3	11.2	11.5	381	608	451	439
13:00	140	3.1	6.0	23.6	25.4	283	120	112	6.0	8.7	6.9	310	557	384	483
6:15	112	1.9	7.0	19.6	21.6	126	115	105	3.8	6.8	4.8	132	623	348	493
11:40	123	2.0	7.0	37.4	32.6	400	113	129	9.0	6.9	6.5	503	589	771	817
20:00	150	2.5	7.0	40.2	37.0	745	139	151	12.8	10.6	11.3	980	574	694	651
11:20	131	2.7	7.0	44.4	41.0	548	143	155	16.1	13.5	14.5	770	552	660	613
14:10	135	2.5	7.0	50.2	46.0	708	135	147	21.0	17.0	18.2	1050	477	589	550
10:40	130	2.8	7.0	50.6	53.0	427	103	98				600	—	—	—
11:10	134	3.0	7.0	40.4	45.0	640	190	171	10.1	12.8	13.0	830	\$775(986)	\$612(779)	\$602(766)
9:20	115	1.6	8.0	33.0	37.8	377	141	123	6.6	8.6	8.6	505	943	724	725
12:30	130	2.4	7.0	27.6	32.0	378	142	123	4.0	6.0	5.2	450	1171	780	899
7:10	117	2.4	7.0	24.4	27.0	241	161	146	2.8	3.8	3.2	200	1165	859	1020
Subject M. (male)															
Subject M. (male)															

TECHNIQUE

Venous and arterial blood samples were taken every ten minutes. Blood was collected from a retention cannula in the median cubital vein, brachial artery or femoral artery. Clotting was prevented in the venous cannula by constant, slow infusion of a dilute heparin-saline solution.

Urine was collected by catheter and the bladder was washed twice with 50 ml. of water plus air after each discharge.

Diodrast was analyzed by Bak, Brun and Raaschou's (5) modification of White and Rolf's (6, 7) method. Inulin was analyzed by Brun's (8) modification of Corcoran and Page's (9) and Miller, Alving and Rubin's (10) method.

Procedure of experiments. The subjects used in this study showed no evidence of cardiovascular-renal disease and were afebrile.

The experiments were carried out in three different ways. Generally, a rather rapid rise in the plasma concentration was first obtained by the intravenous infusion of diodrast solution. The infusion was discontinued when the concentration had reached a sufficiently high level, and the concentration was left to fall spontaneously. In other subjects a large intravenous single injection brought about a high diodrast concentration which was allowed to fall spontaneously.

RESULTS OF EXPERIMENTS

Six experiments were made with simultaneous determination of the diodrast level in arterial and venous blood on rising and falling concentration. Figure 2 illustrates the blood curves plotted for all six experiments. Table I shows the results from two of the experiments (P. L. and M.) in a more detailed form.

During falling plasma concentration the diodrast level is higher in venous blood than in simultaneous arterial blood, whereas the reverse is the case during rising plasma concentration.

Table II shows the average percentage differences between diodrast and inulin concentrations in arterial and venous blood (expressed in per cent of the arterial blood concentration) during spontaneously falling concentrations. In the case of diodrast the differences have been calculated only for the concentrations below the self-depression limit. With regard to diodrast the average difference for all six experiments was 29 per cent. This figure corresponds approximately to what might be expected from theoretical calculations. The arteriovenous difference in concentration will depend partly on the diodrast extraction in the kidneys and partly on the ratio between the "renal"

TABLE II

Comparison of diodrast and inulin concentration differences (in per cent) in arterial and systemic venous blood during spontaneously falling concentrations

Subject	Diodrast	Inulin
	<i>per cent</i>	<i>per cent</i>
H. A. (male)	28	5.8
P. J. (male)	26	7.0
M. (male)	31.3	8.0
A. (male)	10.9	
P. L. (male)	52.0	8.7
Average	28.7	7.4

The values are obtained using the following formula:

$$\frac{P_{DV} - P_{DA}}{P_{DA}} \cdot 100 \quad \frac{P_{INV} - P_{INA}}{P_{INA}} \cdot 100$$

in which P_{DA} and P_{INA} are the arterial plasma concentrations of diodrast and inulin and P_{DV} and P_{INV} are the venous plasma concentrations of diodrast and inulin.

and the "extrarenal" minute volume. Starting from a level below the self-depression limit, where the renal venous blood is almost completely cleared of diodrast, and supposing that one-fifth of the minute volume of the heart passes through the kidneys, we may set up the following equation from which the ratio of arterial concentration to venous blood concentration may be calculated:

$$V \times P_{DA} = 1/5V \times 0 + 4/5V \times P_{DV} \\ P_{DV} = 5/4V \times P_{DA}$$

P_{DA} and P_{DV} are the diodrast concentrations in arterial and venous blood, and V the cardiac output.

In the case of inulin, the renal venous blood will be cleared of only about one-fifth of the quantity present in the arterial blood. Hence the equation for inulin is as follows:

$$V \times P_{INA} = 1/25V \times 0 + 24/25V \times P_{INV} \\ P_{INV} = 24/25V \times P_{INA}$$

where P_{INA} and P_{INV} indicate the inulin concentrations in arterial and venous blood.

In the case of inulin, too, the difference found (7.4 per cent Table II) corresponds fairly closely to what might have been expected.

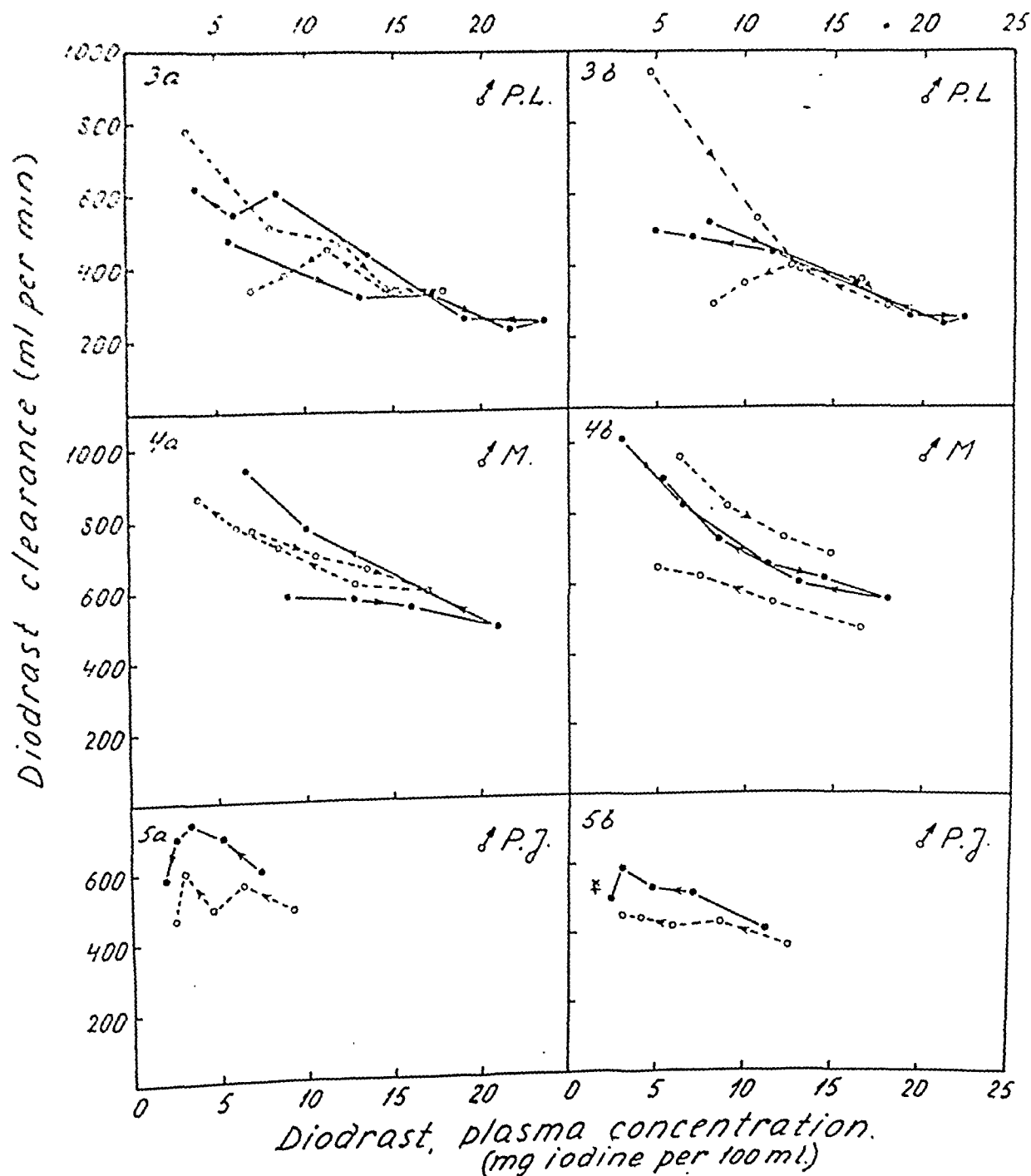
These calculations are approximate, of course, because we do not know the exact ratio between the cardiac output and the renal blood flow. Moreover, we cannot *a priori* be certain that the deposition of diodrast is uniform in all tissues. On the contrary, investigations by Lengemann (11) seem to show that the largest amounts of diodrast are deposited in liver and skin. The blood from the median cubital vein is cutaneous venous blood and

it may be particularly rich in diodrast on falling plasma concentration.

During rising plasma concentration the level in the arterial blood is, as mentioned above, higher than in the corresponding venous blood, partly because a certain amount of diodrast is deposited in the tissues and partly because of the variations in

the distribution of diodrast in the blood. The rate of infusion is the factor determining the arterio-venous concentration difference.

We shall now report the results arrived at by calculating the diodrast clearance on the basis of arterial blood concentration and venous blood concentration, respectively. The diodrast clearance



FIGS. 3-5. DIODRAST CLEARANCE VALUES CALCULATED ON THE BASIS OF ARTERIAL AND VENOUS PLASMA CONCENTRATIONS

Figs. 3a, 4a, and 5a show uncorrected clearance values, and Figs. 3b, 4b, and 5b show clearance values which are corrected for delay time. Solid dots represent arterial, and open circles venous plasma concentrations.

values refer partly to Table I (P. L. and M.), and partly to Figures 3, 4 and 5 (P. L., M., and P. J.).

By using the arterial and venous concentrations and without allowing for delay time we arrive at the results indicated in Figures 3a, 4a, and 5a.

In Figure 3a (P. L.), the "venous clearance" values are seen to be high on rising and low on falling blood concentration, while the "arterial clearances" are low on rising and high on falling blood concentration. On the other hand, it appears that the "venous clearance" (Figure 4a) may be identical on rising and falling blood concentrations, whereas the "arterial clearance" is low on rising and high on falling blood concentration. Finally, it appears from Figure 5a that the "arterial clear-

lay time in normal individuals for different urine volumes. In the present article it will only be pointed out that the delay time must be supposed to be longer than it was previously thought to be (three minutes), (Goldring, Chasis, Ranges and Smith [3]). For urine volumes like those dealt with in our experiments ($1\frac{1}{2}$ to 5 ml. per min.) the delay time is between five and eight minutes.

With allowance for these facts we have calculated the diodrast clearance values indicated in Figures 3b, 4b and 5b, as well as in Tables I and II, which show that the "arterial clearance" values are identical during rising and falling blood concentrations, whereas the "venous clearance" values are low on falling and high on rising blood concentration. It appears from Figure 5b that, after correction for delay time, the "arterial diodrast clearance" values calculated during falling blood concentration are found to be comparable with the diodrast clearance calculated on the basis of a constant plasma concentration curve.

Finally some experiments were made in order to investigate the arterial and the venous plasma concentrations of diodrast using the constant intravenous infusion method. The arterial and the venous plasma concentrations are seen to differ very little (Figure 6). The clearance values calculated on the basis of the arterial plasma concentration were practically identical with those calculated on the venous plasma concentration.

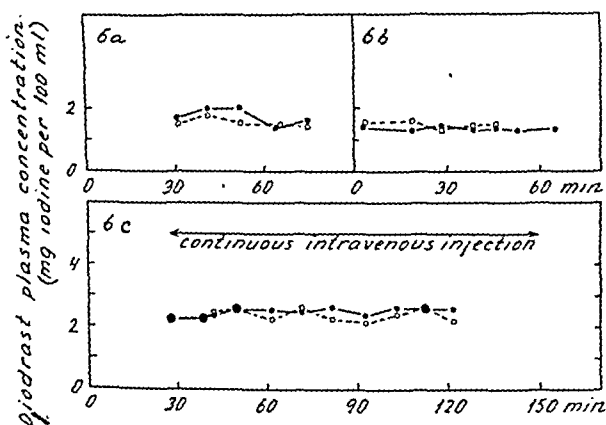


FIG. 6 (a through c). DIODRAST PLASMA CONCENTRATION IN ARTERIAL AND VENOUS BLOOD DURING A CONSTANT INTRAVENOUS INFUSION.

Solid dots represent concentrations in arterial, and open circles concentrations in venous blood.

ance" values are higher during spontaneously falling blood concentration than the "venous clearance." That the diodrast clearance has been calculated on the basis of concentrations above the self-depression limit is naturally of no importance in these subjects, where it was the question of comparing the "arterial" and the "venous" clearance values calculated on simultaneous plasma concentrations, and not of estimating the absolute clearance values.

However, we feel that allowance should be made for delay time in the calculation of the diodrast clearance. In a subsequent article (12) we will publish the results of experiments dealing with de-

DISCUSSION

It appears from the results of the above experiments that the "venous diodrast clearance" values calculated during rapidly falling or rising plasma concentrations are either too low or too high. The "arterial diodrast clearance," on the other hand, presents identical and correct values under the same experimental conditions *provided that allowance is made for delay time*.

We may, therefore, for the present conclude that the cause has been found for the error in the diodrast clearance, when it is calculated using venous blood analyses and falling plasma concentration; the error is due to the difference between the diodrast concentrations in arterial and venous blood.

Foa and Foa (13) calculated the diodrast clearance on the basis of a falling plasma concentration

curve and venous blood analyses, a procedure which, according to the above observations, we cannot recommend. That these writers found the average normal value to be as high as 566 ml./min. is no doubt due to the fact that they made no allowance for delay time. This may in some measure eliminate the error made in diodrast clearance calculation based on rapidly rising and falling plasma concentrations, and may offer an explanation of the fact that in some cases we did not observe low clearance values on falling concentrations.

It has previously been mentioned that strikingly high and irregular diodrast clearance values may be observed within the first part of an infusion period (cf. Figures 1a and 1b), as well as at a later stage of the experiment, whenever there is a great increase in the infusion rate. This may be owing in part to the fact that the tissues are not saturated with diodrast, and consequently, the percentage difference between the diodrast concentrations in arterial and venous blood is large. The plasma concentrations, therefore, used in the clearance calculation are far too low when venous blood analyses are used. Another cause may be the fact that at the beginning of some of our experiments the infusion was irregular, so that sudden and relatively great changes might have occurred in the diodrast concentration in arterial blood without these fluctuations necessarily being reflected to the same extent in the venous blood. Finally the possibility cannot be omitted that actual variations in the renal plasma flow underlie these high and irregular clearance values. ✓

CONCLUSIONS

1. Diodrast clearance values obtained during rapidly falling plasma concentration are erroneously low. This error is due to the fact that significant differences in diodrast concentrations between arterial and systemic venous blood occur during rapidly rising and falling plasma concentrations. Therefore the customary use of peripheral plasma concentration in calculations of the diodrast clearance can lead to large errors.

2. If arterial plasma concentration is used and if correction is made for delay time the diodrast

clearance is independent of rate of change in the plasma concentration.

3. If a constant intravenous infusion is used, the clearance calculation using venous plasma concentration is permissible.

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STUDIES ON PAIN: MEASUREMENTS OF PAIN INTENSITY IN CHILDBIRTH¹

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It is a common observation that patients differ widely in their reaction to childbirth, some women giving evidence of great distress and others maintaining a high degree of equanimity throughout labor. These variations in reaction have led obstetricians to question the existence of pain in certain patients (1), except for the sensations accompanying the surgical procedures often required for the delivery of the baby. In the absence of a quantitative method for measurement of pain intensity, the reaction of the patient has been the principal index of the severity of labor pains.

The introduction of dolorimetry, a laboratory method of measuring painfulness, by Hardy, Wolff and Goodell (2, 3), has made it possible to obtain reliable estimates of spontaneous and experimentally induced pain intensity. This method provides a scale based on the ability of an individual to discriminate differences in the intensities of pain. The technique consists essentially of comparing the intensity of a spontaneous pain with that induced on the skin of the subject by thermal radiation. No clinical application of this method has yet been made, and in applying it to the study of the pain experienced during labor, it was hoped that the patient could make reproducible comparisons of the labor pains with those evoked by the thermal stimulus on the skin. If this proved possible, the intensity of the painful sensations experienced by a patient in the various stages of labor and by different patients, could be compared on a common basis. This would provide a convenient method for the evaluation of the effectiveness and mode of action of analgesic agents administered for the relief of pain during labor.

It is the object of this report to present experimental data on pain intensity, obtained from a study of 13 women in the various stages of labor

before the administration of any analgesic or anesthetic agent.

METHOD

Thirteen normal young women without obvious medical or obstetrical complications cooperated as subjects in these experiments. There were ten primiparas and three multiparas. Nine were private patients who had a definite interest in the investigation as they were either former nurses or wives of physicians or professional men. Special urging was unnecessary to persuade these patients to participate in this study, as either curiosity or desire to be of service caused them to volunteer readily. All patients had one motive in common, namely, to withstand any reasonable discomfort if it would help ease the suffering of future patients. The matter was, of course, discussed with the husband and in instances in which the patient was the wife of a physician the husband remained in the room while the study progressed. In two instances the pain studies were discontinued in the latter part of the first stage of labor at the patients' request. At first private patients were deemed most suitable for this study as it was not clear that discriminating differences in intensity of pain would be a simple matter during the course of labor. Since these patients had no difficulty in making the necessary comparisons, four clinic patients were subsequently included in the study.

Prior to hospital admission in labor, all of the private patients had been given preliminary instruction in the thermal radiation technique of pain threshold measurement (4). The operation of the equipment was explained to them, and they were introduced to the several persons involved in the study who would be present during the investigation. As the authors and a technician remained with the patient during the entire period of observation, it was felt desirable that rapport be established between the patient and the experimental team.

During the first laboratory interview the skin of the dorsum of the right hand was blackened with India ink in four areas, and the pain threshold measured on these test surfaces. The pain threshold was measured by first exposing the blackened skin for three seconds to an intensity of thermal radiation slightly above the pain threshold so that a distinct pain was felt. Then, beginning with a stimulus well below the pain threshold, the radiation intensity was increased through successive exposures until the patient reported a barely perceptible prick at the end of the exposure. This intensity was

¹ This work was supported in part by funds from the Office of Naval Research of the United States Navy.

taken as the pain threshold. It is of the greatest importance that different areas of skin be stimulated in each exposure, and that as few stimuli as possible be given to locate accurately the threshold. An average of four stimuli was usually sufficient to determine the pain threshold within plus-minus ten millicalories per second per square centimeter. None of the patients studied had the slightest difficulty in recognizing the pain threshold.

During this period of instruction the difference in intensity of two pains was demonstrated by exposing the patient to a stimulus evoking a two-dol² pain and then to one evoking a four-dol pain. This was done to impress upon the patient the importance of the intensity aspect of pain as contrasted with its quality and duration, since the experimental pain ostensibly differed from the labor pain in these respects. Most of the patients commented upon the fact that the sensation from the uterus would be different in quality, duration and locale, and, therefore, expressed some doubt as to the possibility of equating the two sensations in terms of intensity. These apprehensions were not borne out by experience as only two of the patients had difficulty in making comparisons, and in these instances the degree of cooperation was not sufficient to continue the observations through the first stage of labor.

The use of the word "pain" in connection with the uterine contraction was avoided in discussing the subject with the patients, and "intensity of contraction" was the descriptive term employed. It was hoped in this way to avoid suggesting to the patient that her labor would be painful.

During labor the technique for measuring pain intensity was as follows: Immediately following a contraction the patient was given a three-second exposure to the thermal stimulus. She made a report as to whether or not the sensation on the back of her hand was more or less intense than that experienced from the uterine contraction. Depending upon this report the intensity of the stimulus was increased or decreased and the test repeated in order to "bracket" the uterine sensation. By having two stimuli, one lower and one higher than the spontaneous sensation, it was possible to find quickly the stimulus which the patient reported to be approximately of the same intensity as her labor pain. Usually three or four stimuli were enough to locate the balance point between the sensation from the uterus and that from the pain apparatus, but if more stimuli were required a rest period of about ten minutes was allowed before resuming tests. Care was taken throughout not to overstimulate any area of blackened skin and thereby produce local hyperalgesia.

It was repeatedly observed that the intensity report was the same whether the comparison of sensations was made at the height of a contraction or in the interval between contractions. As there was more time available between contractions comparisons were usually made then, adjusting the thermal stimulus to produce a sensation equivalent

² "Dol" is the term used to denote the unit of painfulness, and has a value of approximately one-tenth the intensity of the maximal pain.

in intensity to that evoked by the previous contraction. Two measurements were made and if the reports agreed within one dol they were averaged and used as the point to be plotted. Single test reports were used only at the highest pain intensities, because of tissue damage resulting from the strong thermal stimuli. Large variations in reports of intensity (three to five dols) occurred when the patient was distracted or not cooperating, and were observed in both patients who requested that the study be discontinued. This possibility had been anticipated since erratic reporting had been observed in a previous study of medical students (3). In spite of the clinical procedures necessarily carried out during the course of the pain study, the fatigue of the patient, and other disturbing circumstances that could not be controlled or eliminated, distraction resulting in erratic estimates was not an important factor. Reproducibility of the measurements within plus-minus one dol was taken to be the criterion of the excellence of the data.

Continued emphasis was placed during labor upon the *intensity* of the sensation as distinct from its quality and duration, because the patients frequently stated that the thermal pain was not "comparable" to the labor pain. The difference between the experimental pain and the labor pain may have caused some difficulty in making accurate comparisons. However, repeated comparisons during labor showed only variations of approximately plus-minus one dol, and as the limit of discrimination is plus-minus one-half dol this shows that the patients were making satisfactory appraisals.

It is important to keep in mind that as the severity of labor pain increases, thermal stimuli of correspondingly higher intensity must be administered and tissue damage will be produced when the comparisons extend beyond eight dols.

Pain threshold measurements were made throughout the entire period of study, in the intervals between contractions. It has been previously shown that an existing pain will cause an elevation of the pain threshold elsewhere in the body (4), and for this reason pain threshold measurements were not made during a contraction.

As soon as the patients were admitted to the hospital they were placed in a private labor room for study. One of the experimental team remained with the patient at all times, and pain tests were made every half hour or when there was any change in the patient's obstetrical status. The majority of the measurements were made in the first stage of labor, although two patients were observed in the second stage, and two in the fourth stage (5). No measurements were made in the third stage. None of the patients had had any analgesic or anesthetic during the time they were studied, although subsequent to the pain studies nine had analgesics and 11 had anesthetics for the delivery. If at any time during the study a patient requested an analgesic it was administered and the pain studies were terminated. This accounts for the fact that most of the patients were studied in the early and middle parts of the first stage of labor.

Measurements were made of the frequency and duration of uterine contractions by manual palpation of the

subject's abdomen. Cervical dilatation was estimated by rectal examination and in some instances confirmed by sterile vaginal examination.

In four patients records were made of the uterine contractions by means of a pneumograph strapped to the abdomen. It was not anticipated that the excursions of the pneumographic needle would be proportional to the intensity of the uterine contraction, but the duration of contraction and the interval between contractions could be more accurately determined in this way than by manual palpation of the abdomen. The patient was given a signal magnet key to press during the time she felt pain, as it was desirable to know whether or not the pain outlasted the contraction. The patient was also asked to give an estimate of the intensity of her pain each five seconds during the contraction. The reports were made in terms of fractions of the intensity of a recently measured uterine pain. This type of procedure for estimating pain intensity had been proved successful in the study of headache and other pains (6, 7).

In the course of all studies careful notes were kept as to the patients' reactions, such as crying, complaining, sweating and degree of alertness and cooperation in the pain studies. Such notes were used to arrive at an estimate of the patient's reaction to her pain.

RESULTS

Figure 1 shows the results obtained on the first patient studied. This individual was a primipara, a professional woman of high intelligence, who wished to experience labor without analgesia, because of her interest in Read's publications on natural childbirth. Studies were begun five hours after onset of the first pains, at which time labor was still mild, and the pain intensity averaged approximately three dols. The cervix was three centimeters dilated; uterine contractions occurred at five-minute intervals and lasted approximately 30 seconds. During the next five hours, as the cervix dilated gradually, the pain increased steadily, reaching an intensity of eight dols, and the contraction interval decreased to one minute.

The subject was calm throughout the course of her labor except for obvious tenseness. However, after the pain had reached eight dols (a severe pain) she asked for an analgesic. This was ad-

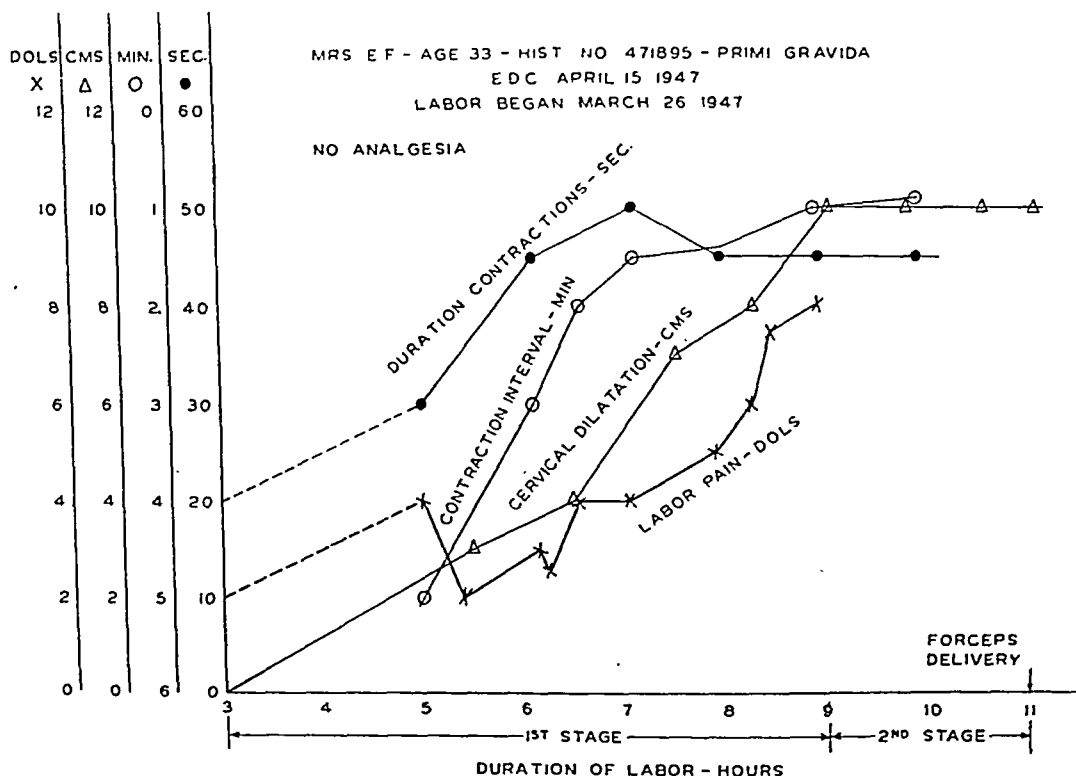


FIG. 1. PAIN CHART OF MRS. F

Abscissa: Duration of labor in hours.

Ordinates: Dots represent duration of contraction in seconds; circles—contraction interval in minutes; triangles—cervical dilatation in centimeters; and crosses—pain intensity in dols.

ministered in the form of nitrous oxide during the second stage.

The second patient was a multipara who had had an unhappy obstetrical history characterized by six spontaneous abortions and only one living child. When the study began her pains were moderately severe, the cervix was closed and the contraction interval was about four minutes. In the next two hours the pain rose very rapidly to the extreme range above nine dols, as shown in Figure 2. The cervix dilated rapidly, and the contraction interval shortened in proportion. Two measurements were made on this patient during the second stage of labor, although the patient had been repeatedly warned of the danger of blistering. Yet she wished to cooperate fully as an expression of her gratitude in having a term pregnancy and insisted on having the tests made. A pain intensity of $10\frac{1}{2}$ dols was measured. This is the most intense pain which can be experienced. Increasing the intensity of the thermal radiation can cause

no further increase in pain perception (2). Second degree burns were inflicted upon the hands of this patient by the four tests made at levels higher than nine dols. Delivery was accomplished under general anesthesia.

Figure 3 shows a composite study of the 13 patients. As each determination represents not less than eight tests for pain intensity, the 55 measurements are the result of over 400 test readings. These 55 measurements represent an average of about four per patient during the various stages of labor. Since the first stage of labor had a variable duration for each patient, the time scale is expressed in quarters in order to obtain a common basis for comparison. It will be observed that there was a gradual increase in pain intensity as delivery approached, at which time pain reached nine and even $10\frac{1}{2}$ dols. The dashed line in the figure represents an approximate average of observed values in the first and second stages of labor. In the case of subject H, whose values lie

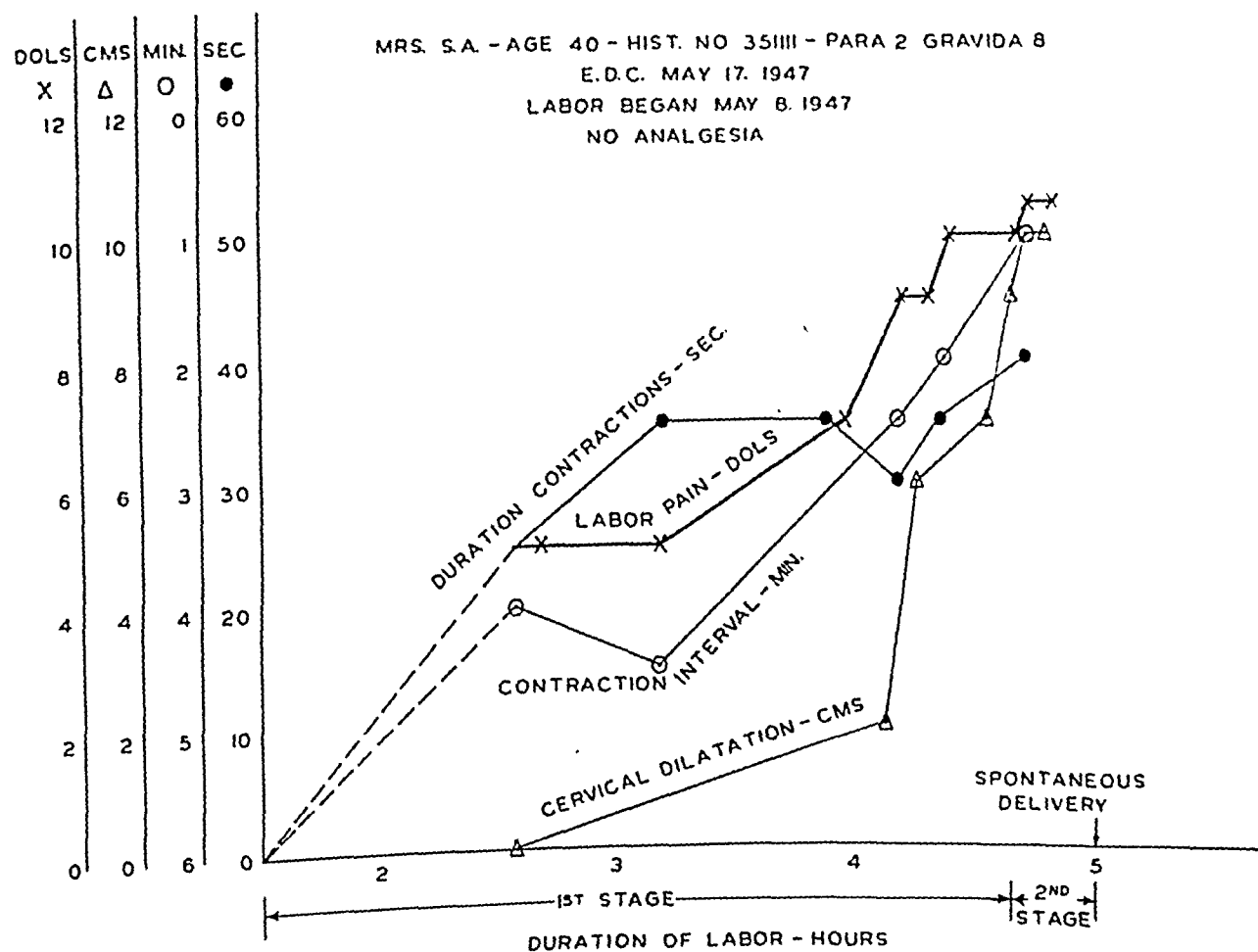


FIG. 2. PAIN CHART OF MRS. A
Symbols the same as in Figure 1.

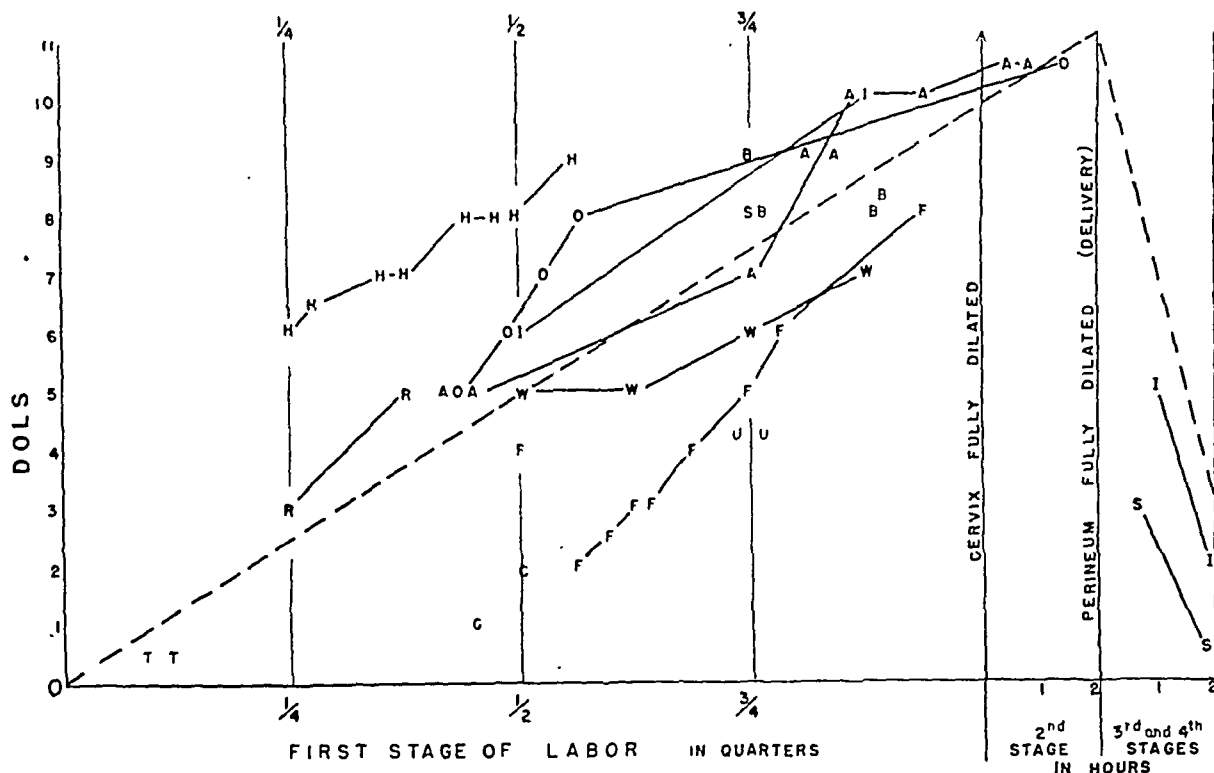


FIG. 3. PLOT OF 55 DETERMINATIONS OF PAIN INTENSITY ON 13 PATIENTS DURING FIRST, SECOND AND FOURTH STAGES OF LABOR

Time scale of first stage expressed in quarters of its total duration.

quite far above the dashed line, it is to be remarked that the progress of her pains was parallel to the average line, but subsequent to the last determination shown she received a considerable amount of an analgesic which may have prolonged her labor.

The intensity of the after pains was measured in only two patients who had received no analgesia in labor and no anesthesia for delivery. These pains were moderate, three to five dols, a half hour after delivery, and decreased to threshold levels two hours later.

Figure 4 shows typical uterine contractions as recorded on the pneumograph. "A" is the recorded curve of contraction; line "B" shows the duration of pain as recorded by the patient when she pressed the key of the signal magnet; and curve "C" is the estimated intensity of pain. The first contraction was recorded in the early part of the first stage of labor, the second during the middle of the first stage, and the third curve during the second stage. It will be seen that the pain in each case was of shorter duration than the contraction, which is in contrast with the findings in

dysmenorrhea. Chassar Moir (8) has reported that in cases of dysmenorrhea the pain outlasts the uterine contraction and from this observation he suggested that the pain of dysmenorrhea might be attributed to hypoxia in the uterine muscle. It will be noted, too, that the stronger pains represent a greater proportion of the time of contraction than do the weaker pains, the range being from 15 per cent to 95 per cent of the total contraction time, depending upon the intensity of the contraction. This indicates that for weak contractions the pain threshold is reached more slowly than for stronger contractions.

The duration of contractions varied considerably from time to time in the same patient in various stages of labor, and showed no tendency to increase as delivery approached. This is in agreement with the observations of Murphy (9) who reports a decrease in average contraction time as labor advances.

DISCUSSION

The first questions to be answered were whether or not the method of dolorimetry was applicable to

the measurement of pain intensity in labor, and, if so, what were the limitations of the method.

The more than 400 trial tests resulting in 55 successful measurements of pain intensity on 13 patients establishes the method as a practical one for this type of investigation. However, this study does not attempt to be a complete survey since it does not include examples of the many types of labor and dystocia that may be found in the obstetrician's practice. Measurements can be made easily on any patient who can cooperate. The effects of distraction and lack of cooperation can be recognized by the large and rapid variations in estimates of pain intensity. Although the data are obtained from subjective comparisons of pain intensity, the test instrument affords an evaluation of the patient's report over which the patient has no control.

The most important limitation of the method is the inflicting of burns when high pain intensities are measured. A second limitation is that measurements cannot be made in the face of distraction and lack of cooperation. The patient must be willing and able to make comparisons of her painful sensations with those induced by thermal stimuli.

1. Pain intensity

In every patient studied the pain intensity increased as the labor progressed (Figure 3). Although few observations are available during the first quarter of the first stage of labor it can be safely assumed that these pains were of a mild intensity, averaging probably not more than one or two dols. Pain in the second quarter of the first stage was moderate, averaging between three and five dols. During the third quarter the pain of most patients averaged between five and seven dols, which is a severe pain. It was at this point that most patients asked for relief. In the last quarter, the pain was between seven and ten dols. The second stage of labor was characterized by the most severe pains, $10\frac{1}{2}$ dols, accompanying the "bearing down." After delivery the pains observed in the fourth stage of labor were again of mild intensity.

An approximate correlation could be made between the intensity of the pain and the extent of the cervical dilatation. Thus, when the patient was experiencing pain of ten dols it was found that the cervix was fully dilated, and if the pain was of one or two dols' intensity the cervix was observed

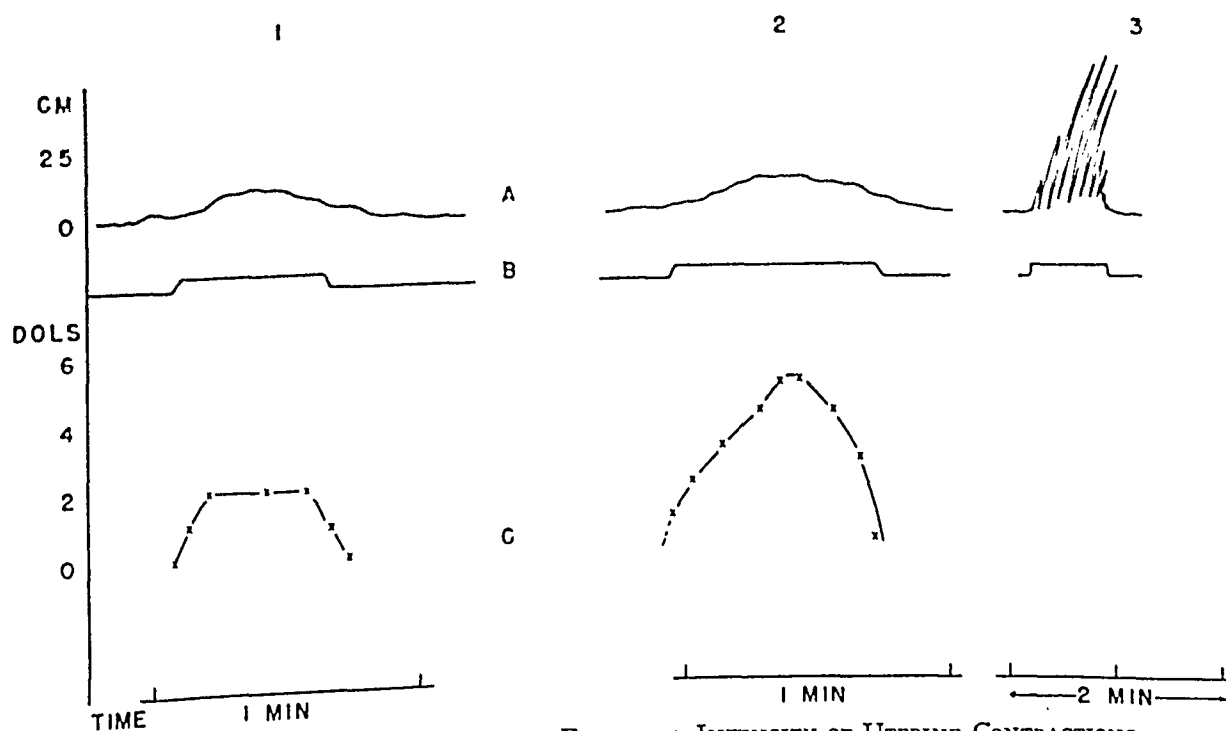


FIG. 4. PNEUMOGRAPH RECORDING AND ESTIMATED INTENSITY OF UTERINE CONTRACTIONS

- A. Typical uterine contractions as recorded by a pneumograph strapped to patient's abdomen.
 B. Duration of corresponding pain recorded by signal magnet.
 C. Estimates of corresponding pain intensity.
 1. Early first stage. 2. Middle of first stage. 3. Early second stage.

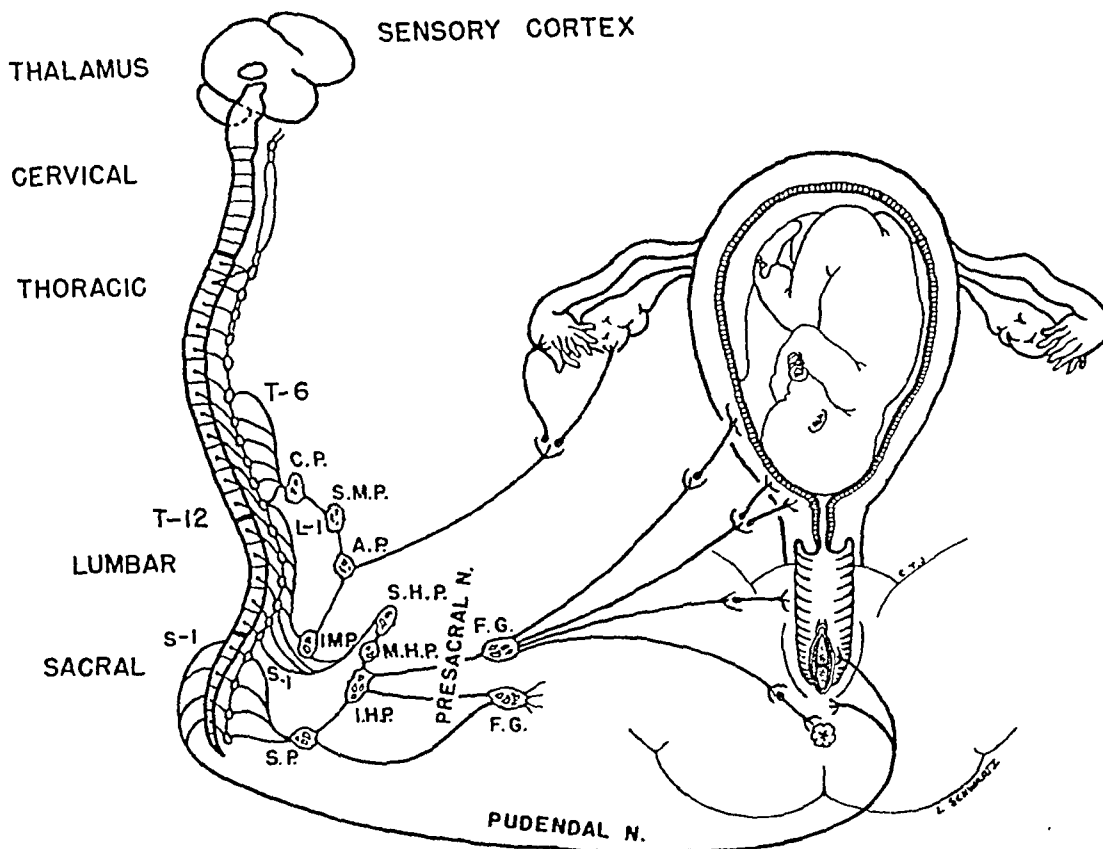


FIG. 5. DIAGRAM OF THE PAIN PATHWAYS OF IMPORTANCE IN CHILDBIRTH

First stage of labor: Visceral afferents arising in plexi enervating uterus, cervix and vagina; pathway: Frankenhouser's ganglion (F.G.) into hypogastric plexi (I.H.P., M.H.P., and S.H.P.) thence through the celiac plexus (C.P.), the superior and inferior mesenteric and aortic plexi (S.M.P., I.M.P., and A.P.) and the sacral plexus (S.P.) into spinal outflow from T6 to S4.

Second stage of labor: Visceral afferents plus somatic afferents via the pudendal nerve.

to be almost closed. However, a much more quantitative relationship appeared to exist between the pain intensity and the interval between contractions.

The relationship observed between the contraction interval and the intensity of pain provides a simple method for computing the pain from measurements of this interval. Thus,

$\text{Dols of pain} = 10.5 - 1.5 \times \text{contraction interval in minutes.}$

This formula may not apply generally, of course, to all cases, although it has been observed to be valid in our series of patients without analgesia.

2. Character and origin of pain

During the early part of the first stage of labor the pain was of short duration and of a dull aching

quality, and was associated with a uterine contraction of relatively low intensity (see Figure 4, Curve 1). The visceral structures involved in producing this pain are deep and noxious impulses probably reach the central nervous system via the sympathetic and parasympathetic afferents (10) as diagrammed in Figure 5. As the cervix dilated and the uterine contractions became stronger the pain assumed a sharper quality. It had been noticed in other studies of pain intensity that bright, burning pain and dull, aching pain have a tendency to become similar. That is, a burning pain had an aching quality and an aching pain became sharp (3). This was also true of labor pains.

The ceiling pain was not reached until the beginning of the second stage at which time the somatic perineal structures were involved. These

superficial structures are innervated chiefly by the pudendal nerve (10), as shown in Figure 5, and the most intense pains are caused by the stretching and tearing of these tissues, with accompanying stimulation of the perineal reflex and considerable increase in intrauterine pressure (10). Curve 3, Figure 4, shows a recording of the onset of the reflex which is apparently inhibited periodically, possibly by the intense pain. Superimposed on the low uterine component of the pressure wave were spikes of great pressure from the perineal reflex. The duration of the pain at this time was almost as long as the contraction.

Figure 6 shows a plot of the pain intensity in dols against the uterine contraction in minutes. The connection between the contraction interval and uterine effort has been established by the tocographic measurements of Murphy (9) and others (11), and while it cannot be assumed that the tocographic measurements are necessarily representative of intrauterine pressure, they appar-

ently do represent the uterine hardness and thereby offer a measure of the uterine spasm (12). The uterine pressure in millimeters as estimated from Murphy's work is plotted to the right in Figure 6, and it can be seen that the pain intensity in dols is also proportional to the uterine pressure in millimeters, measured on Murphy's tocograph. This evidence, as well as the clinical observation that dilatation of the cervical canal causes severe pain, supports the conclusion that the pain in the first stage of labor is largely due to the distention of the internal os and the cervical canal as a result of the increased intrauterine pressure caused by uterine contractions.

3. Reactions to pain

During the early hours of labor there were generally few complaints and the patients were alert and interested in the study. There was no sweating. Two patients did not fall into this pattern. Mrs. O soon began to show hostility to the entire experimental team in spite of expressing many

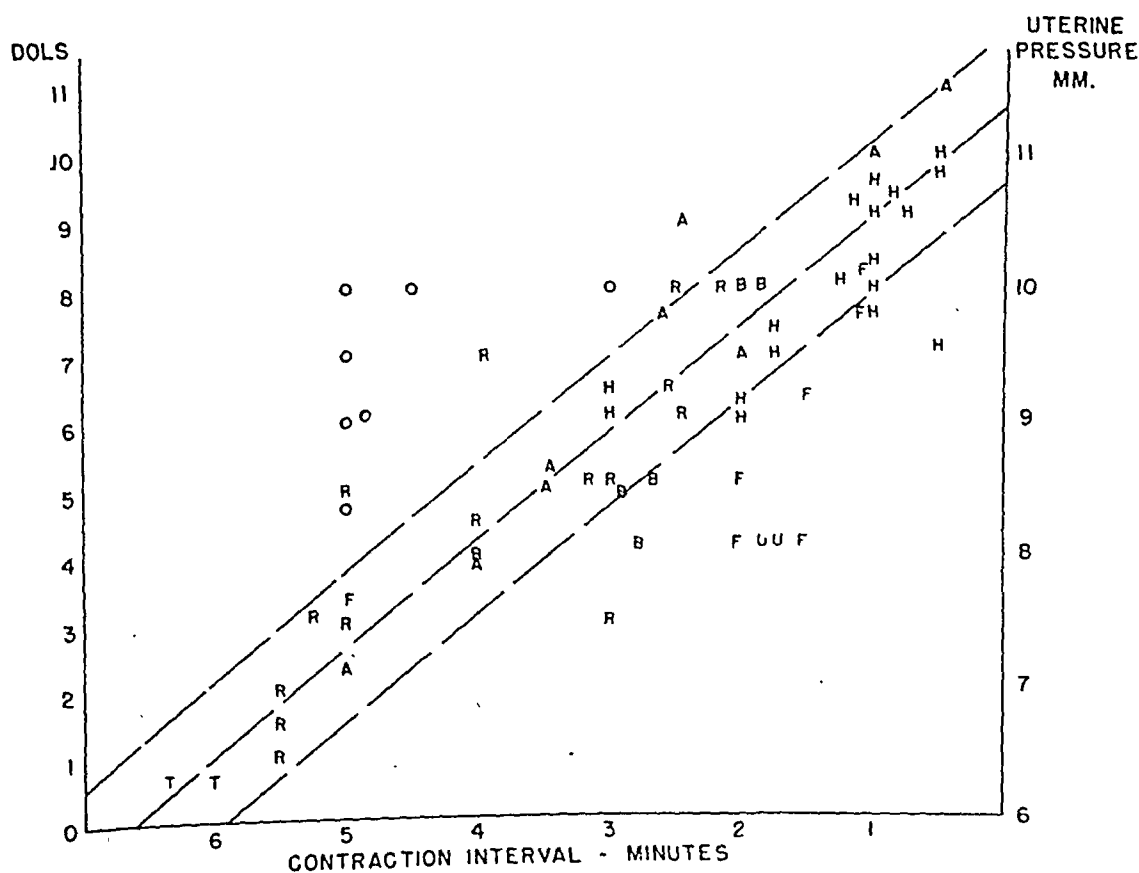


FIG. 6. PLOT OF ESTIMATES OF PAIN INTENSITY (DOLS) AGAINST LENGTH OF INTERVAL BETWEEN CONTRACTIONS (MINUTES)

Ordinate at the right of diagram represents Murphy's tocographic measurements of uterine pressure.

times prior to admission her desire to participate in the study. Cajoling and persistence made it possible to carry on the study and in spite of this difficulty the pain measurements obtained were in the range of those for the more cooperative patients. Mrs. U cried and complained with vigor from the outset although her pain measurements indicated only two to four dols. This patient succeeded in becoming so excited that no further observations could be made in her case.

Late in the first stage 11 of the 13 patients were sweating and crying with each contraction, and were cooperating only with difficulty. Pain was severe at this time, seven to ten dols. One patient, Mrs. A (see Figure 2), gave little evidence of her pain. As a multipara her labor was short and this may account for her restraint in the face of pains measured at eight to ten dols. Fatigue was as important as pain intensity in bringing about a breakdown in the restraint most of the patients maintained over their actions. Thus, in her short labor, Mrs. A gave less visible evidence of pain at eight dols than did the other patients with longer labors at four dols.

The evidence of this study is that the reactions of the patient depended partly on the pain and to a greater extent on fatigue, anxiety, and, in some cases, hostility, whereas the pain intensity as measured was related to the uterine activity and dilatation of the cervical and perineal tissues. It seems important, therefore, to make a clear distinction between the intensity of the pain perceived by the patient and visible evidences of reaction to pain, such as complaining, groaning and crying out. These findings bear out the clinical impression regarding the psychological factors affecting the behavior of patients in labor. However, by measuring the intensity of the pain it is now possible to evaluate more fully the effects of pre-conditioning (as in "natural childbirth") and reassurance, as well as the action of analgesia upon pain sensation in the obstetrical patient. Further studies are planned to include an appraisal of suggestion and hypnosis.

SUMMARY AND CONCLUSIONS

1. Four hundred test readings, resulting in 55 measurements of pain intensity, were made on 13 patients during the first, second and fourth stages of labor without analgesia. The measurements

were made by comparison of the labor pains with a pain of standard intensity which was produced by a three-second exposure to thermal radiation on the dorsal surface of the right hand. The pain evoked on the hand had been previously standardized into $10\frac{1}{2}$ units of painfulness ($10\frac{1}{2}$ dols) between the threshold pain and the most intense pain which can be experienced (ceiling pain).

2. The pain intensity was observed to change with the progress of labor as follows:

- a. First stage—

First quarter—threshold to two dols

Second quarter—three to five dols

Third quarter—five to seven dols

Fourth quarter—seven to ten dols.

- b. Second stage—Ceiling pain, ten to ten and one-half dols.

- c. Fourth stage—Intensity from three to five dols to near threshold value in about two hours after delivery.

3. The intensity of the pain in the first stage of labor was roughly proportional to the extent of cervical dilatation and inversely proportional to the duration of the interval between uterine contractions. Pain intensity can be estimated approximately by the formula:

$$\text{Dols} = 10.5 - 1.5 \times \text{contraction interval in minutes.}$$

4. The duration of pain was 15 per cent of the contraction time for the weak contractions characteristic of early labor, and 95 per cent of the contraction time at the beginning of the second stage of labor.

5. The pain threshold was measured in the interval between contractions in the first, second and fourth stages of labor. It was within the normal range for all the patients who had received no analgesia or anesthesia.

6. The intensity of the pain experienced by the patient could not always be evaluated on the basis of her reaction, nor correlated with her apparent distress. Distinguishing between the intensity of the pain perceived by the patient and the patient's reaction has been made possible by measurements of pain intensity and pain thresholds.

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METHODS FOR MEASURING THE ACTIVITY OF COMPONENTS OF THE STREPTOCOCCAL FIBRINOLYTIC SYSTEM, AND STREPTOCOCCAL DESOXYRIBONUCLEASE¹

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Studies of the effects of streptokinase and desoxyribonuclease from hemolytic streptococci on certain pathological processes, reported in an accompanying paper (1), necessitated the development of quantitative methods suitable for routine laboratory assay of the various substances known to affect the streptokinase-plasminogen (streptococcal fibrin-lysing) system. Methods were devised, or earlier methods modified, for the routine assay of streptokinase (streptococcal fibrinolysin), antistreptokinase (antifibrinolysin), serum inhibitor (serum trypsin inhibitor), and plasminogen (serum protease, serum fibrinolysin, serum trypsinase).

MATERIALS

1. *Crude streptokinase*:² The method used for production of streptokinase is based on one previously published (2), but differs principally in the use of ethanol as a precipitant rather than ammonium sulfate.

Cultures of Group C strain H46A are grown in the presence of high glucose concentration, employing intermittent neutralization with NaOH in the manner previously reported (2). The yields of streptokinase and desoxyribonuclease from a typical culture are presented in Figure 1. It will be noted that the peaks of enzyme production coincide with the peak of glucose utilization. As incubation is continued, both streptokinase and desoxyribonuclease levels decline. For this reason it is best to begin harvesting of the culture shortly before glucose utilization ceases.

At the end of the growth period the cells are removed by Sharples centrifugation at about 40,000 rpm. The supernatant is adjusted to pH 3.8–4.2 by the addition of glacial acetic or hydrochloric acid. Cold ethanol at -8°C is added to the supernatant at room temperature to a final concentration of 40–50%. After standing overnight in the cold, the practically clear supernatant is siphoned off and the flocculent precipitate, which contains the streptokinase and desoxyribonuclease, is collected by centrifugation at 2,000–3,000 rpm. The precipitate is dissolved in borate buffer with the aid of alkali, adjusted to pH 7.8–8.0, and lyophilized. This precipitate contains the cells not sedimented by Sharples centrifugation, vari-

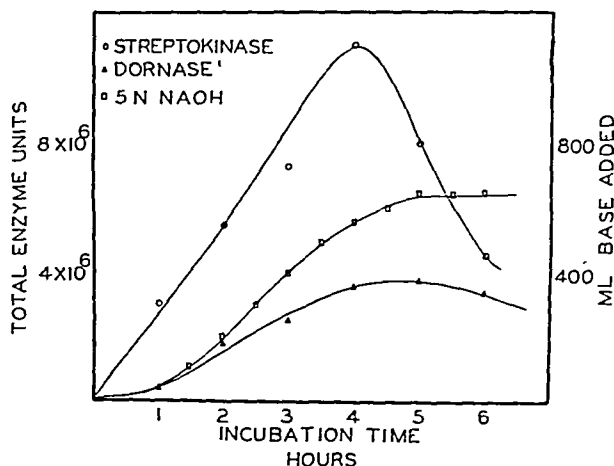


FIG. 1. RELATION OF STREPTOKINASE AND DESOXYRIBONUCLEASE PRODUCTION TO GROWTH (EXPRESSED AS ML. 5N NaOH ADDED TO NEUTRALIZE ACID FORMED)

¹ Desoxyribonuclease.

ous streptococcal proteins, some media components, nucleic acids, streptokinase and desoxyribonuclease.

2. *Purified streptokinase*: The purification procedure is a modification of an earlier method (3). A 2–5% solution of crude streptokinase is made

¹ This study was supported in part by a grant from the Life Insurance Medical Research Fund and the Ralph B. Rogers Rheumatic Fever Fund.

² We are greatly indebted to Dr. Benjamin Carey and Mr. J. N. Adam, Jr. of the Lederle Laboratories Division of the American Cyanamid Company for supplying large quantities of crude streptokinase for these studies.

in borate buffer, pH 7.8–8.0. Cells and insoluble debris are spun out at 16,000 rpm in a refrigerated angle centrifuge. Occasionally a significant portion of the activity is also spun down by this treatment. If this is the case, the precipitate is resuspended and made alkaline to thymol blue (above pH 9). The solution is then adjusted to pH 7.8–8.0 and centrifuged. Following this treatment the activity remains in solution. The supernatants are pooled and diluted with distilled water to a nitrogen content of about 0.25–0.50 mg./ml. and sufficient Pillimer's acetate buffer is added (4) to reduce the pH to 5.6. Sufficient 1% protamine³ solution in distilled water is added to cause the formation of a flocculent precipitate (usually about 1 ml./5–10 mg. nitrogen). The protamine precipitate is spun out in a refrigerated angle centrifuge at 4,000–5,000 rpm. The supernatant should show no further precipitation on testing with protamine, but care should be used to avoid any great excess of protamine, since it will complicate later purification steps. The supernatant is cooled to about 0° and ethanol at –10° to –20° is added to a final concentration of 20%. On standing overnight at –8° to –10° a flocculent precipitate forms which is spun off in an angle centrifuge in the cold. The precipitate is dissolved in borate buffer, pH 7.8–8.0. Some insoluble material remains in suspension, but in most cases little activity is associated with it; and it can be removed by centrifugation, resulting in little or no decrease in total activity, but a decrease of about 50% in total nitrogen. The clear solution usually contains between 100 and 180 units of streptokinase per microgram of nitrogen. Recovery usually represents about 25% of the original activity of the culture, some 50% of the original activity being lost during the initial Sharples centrifugation. Desoxyribonuclease, which has accompanied streptokinase in the previous purification steps, is separated from streptokinase by the 20% ethanol fractionation.

³ Several sources of protamine have been tested. In our experience, consistently good results are obtained with Squibb protamine. Protamines from other sources often fail to effect purification, and are less efficient in that much larger quantities are necessary to precipitate inert materials. We wish to express our gratitude to E. R. Squibb and Sons for generous gifts of protamine.

Practically all of the desoxyribonuclease activity remains in the supernatant.

The purified streptokinase is sterilized by filtration through Seitz or Sela filters, lyophilized and stored in the refrigerator. Solutions remain stable for several weeks if not too dilute and if kept in the cold.

3. *Streptococcal desoxyribonuclease*: As mentioned in previous reports (5–7), streptococcal cultures contain a potent desoxyribonuclease. In the original method of purification (2) using ammonium sulfate, both enzymes were found in the same fraction. However, they are readily separated since streptokinase is precipitated at pH 5.6 by 20% ethanol or 50% ammonium sulfate, while the majority of the desoxyribonuclease remains in solution. In the present study, desoxyribonuclease was prepared by bringing the 20% ethanol supernatant of the above protamine-purified streptokinase precipitate to 40–50% ethanol. The precipitate which forms overnight in the cold is collected by centrifugation and dissolved in the gelatin-magnesium-veronal buffer recommended for desoxyribonuclease titration by McCarty (8). The preparations usually contain insoluble material, with which is associated some of the desoxyribonuclease activity. The clear supernatant obtained by centrifugation of the material, however, contains a significant proportion of activity.

Up to the present time methods have not been perfected for the quantitative isolation of the enzyme from streptococcal cultures and recovery represents only a fraction of the amount in the

TABLE I
Fractionation of streptokinase and desoxyribonuclease from streptococcal cultures

Fraction	Total units	
	Streptokinase	Desoxyribonuclease
(1) original culture	1.75×10^6 units	5.25×10^4 units
(2) after Sharples centrifugation	1.5×10^6	4.1×10^4
(3) alcohol precipitate of culture supernatant	$1.2 \times 10^{6*}$	3.0×10^4
(4) 20% alcohol ppt. of protamine supernatant	1.38×10^6	0.1×10^4
(5) 40% alcohol ppt. of (4) supernatant	0.01×10^6	1.8×10^4

* This value is somewhat lower than the true value because sufficient time for reversal of the inactivation, which occurs in the neighborhood of pH 5 (3), was not allowed before titration.

original culture. This loss is due apparently to the instability of the enzyme.

In Table I are presented the data obtained in a typical purification experiment.

4. *Bovine fibrinogen*: For reasons to be discussed later, human fibrinogen preparations were considered unsuitable for assay purposes. Armour's Fraction I of bovine plasma,⁴ a commercial product, has been found to be quite suitable for our purposes, since it contains no antistreptokinase, serum inhibitor or plasminogen activatable by streptokinase. The dry powder is stored in the refrigerator and dissolved in borate buffer, pH 7.4-7.6, in appropriate concentrations before use. Solutions are reasonably stable and generally remain usable for six to eight hours if kept cool.

5. *Plasminogen*: No satisfactory preparation of plasminogen is available. The purer preparations with which we are familiar, such as Howard Fraction III-2,3 (9), contain plasmin as well as plasminogen and proteolytic products of fibrin. The cruder preparations, prepared by salt fractionation (2), isoelectric precipitation (2, 10) or ethanol fractionation, such as Harvard Fraction III (11), contain a variety of substances, some of which undoubtedly influence the streptokinase-plasminogen reaction.; However, reproducible results have been obtained in these laboratories by using a single lot of Harvard Fraction III.⁵ For use, the material is made up in borate buffer in a concentration of 0.25%. The solutions must be used immediately, and kept in an ice bath, because some spontaneous plasmin activity will develop in a few hours. However, when used as outlined, we have noted no variation in the plasminogen titer of the dry material in over a year.

6. *Standard antistreptokinase*: A pool of Harvard Fraction II-III was fractionated by Harvard method 9 (12). Fraction II, the antibody fraction, was lyophilized and stored in a vacuum desiccator.⁶ Assay of the material with strepto-

kinase indicated that 1 mg. would inhibit the activity of 10 units of streptokinase, when tested by the method outlined below. The material was assigned, therefore, a value of 10 antistreptokinase units per mg., and used throughout these studies as a primary standard to check the constancy of the streptokinase unit and of the antistreptokinase titrations.

7. *Crystalline trypsin*: A solution of commercial crystalline trypsin, obtained from the Plaut laboratories, is prepared in N/400 HCl. The stock solution contains 0.1% of the dry powder, of which about 50% is magnesium sulfate. The concentration of active trypsin in the solution is determined by titration with soybean inhibitor, as outlined below, and appropriate dilutions for use, usually containing 20 μ g. of active trypsin per milliliter, are made up in borate buffer immediately before use.

8. *Crystalline soybean inhibitor*: A stock solution of Kunitz' crystalline inhibitor⁷ (13), containing 2 mg./ml. of inhibitor, was prepared in borate buffer. Appropriate dilutions are made up in borate buffer, pH 7.4-7.6, before use.

9. The preparation of other reagents, such as borate buffer and gelatin buffer, have been detailed in previous reports (2, 14).

METHODS

1. *Streptokinase assay*: Many methods have been described for streptokinase assay, utilizing clots prepared from human fibrinogen (15-18). These methods depend on the presence of plasminogen as a contaminant of the fibrinogen preparations, since, as Milstone (10) has shown, highly purified human fibrinogen will not lyse in the presence of streptokinase unless another serum factor (lysin factor, plasminogen) is added. In our experience, methods utilizing human fibrinogen are unsuitable for long-term routine use. The principal disadvantage is the variation from batch to batch in the fibrinogen-plasminogen ratio obtained with different methods of prepa-

Physical Chemistry of the University of Wisconsin for making facilities available and for help in the preparation of various plasma fractions.

⁷ Dr. M. Kunitz kindly supplied us with a preparation of crystalline soybean inhibitor for use as a standard, and made available his assay methods in advance of publication.

⁴ We are indebted to Dr. J. B. Lesh of Armour Laboratories for supplying the Fraction I used throughout this study. Fraction I is an ethanol fraction of bovine plasma and consists largely of fibrinogen.

⁵ We are indebted to Dr. Dwight Mulford, Division of Biologic Laboratories, Massachusetts State Department of Health, for supplying a large lot of Fraction III isolated from a single pool of plasma.

⁶ The author wishes to express his indebtedness to Dr. J. W. Williams and associates of the Department of

ration, and even with the same method. For example, in our experience ethanol fractions of plasma, such as Harvard I, contain less plasminogen than do the salt fractionations previously used in this laboratory (2), as shown by the fact that clots treated with streptokinase will lyse much more readily if the fibrinogen is prepared by salt fractionation rather than the ethanol. Also, human fibrinogen preparations are unstable, due to the spontaneous conversion of plasminogen to plasmin. Another difficulty in the use of human fibrinogen is the presence of variable amounts of antibody globulin in the preparations. While this occasions no particular difficulty with most samples of normal plasma whatever the method of fractionation, we have encountered occasional lots of plasma with such high titers of antistreptokinase that they were unusable.

For these reasons, we have modified the test by the substitution of clots prepared from bovine fibrinogen (Armour's Fraction I). The bovine fibrinogen contains no plasminogen or plasmin, as shown by the fact that such clots, without added protease, will remain stable for days in the presence or absence of streptokinase. Antistreptokinase and inhibitor titrations indicate the absence of these substances also. As a source of plasminogen we have used a single large lot of Harvard Fraction III. The fibrinogen concentration used is 0.1%, as in previous reports, and the concentration of Fraction III is adjusted to give a streptokinase unit equal in value to the one previously employed in this laboratory (3). The streptokinase assay is performed as follows:

- 0.1 ml. streptokinase dilution in gelatin buffer
- 0.4 ml. bovine fibrinogen, 0.25% in borate buffer
- 0.5 ml. Fraction III, 0.25% in borate buffer.
(This solution must be prepared just before use, and should be kept on ice)
- 0.1 ml. Lederle Hemostatic Globulin,⁸ diluted 1:3 in borate buffer.

⁸ Lederle Hemostatic Globulin has recently become unavailable. Parke-Davis bovine thrombin may be substituted as well as Upjohn thrombin. They are less stable than Hemostatic Globulin when in solution in borate buffer, and solutions must be titrated frequently to determine the minimum amount necessary to form a firm clot. These preparations are more stable in glycerol solution, but we have not used glycerol solutions.

The tubes are incubated at 35° and the lysis time of each tube of the series noted. The dilution lysing the clot in 10 minutes contains one unit and is determined by interpolation as outlined in a previous report (3).

2. *Antistreptokinase*: The antistreptokinase unit is defined as the amount of antibody required to inhibit 1 unit of streptokinase to such a degree that it will not activate the plasminogen sufficiently to cause lysis of the standard test clot in 30 minutes. Other methods of antistreptokinase assay have been presented (15, 18-20) but no general agreement has been reached on a method. We have thought it preferable to base the antibody unit on the method of streptokinase titration used in this laboratory. In order to have a primary standard of reference throughout the study, a lot of Fraction II, the gamma globulin fraction of human serum, was prepared and lyophilized. This preparation contained 10 antistreptokinase units per mg., and has been used as a reference standard for streptokinase and antistreptokinase titrations. Standardization of streptokinase with standard antibody is performed as follows:

- 0.5 ml. streptokinase dilutions in gelatin buffer, pH 7.6-7.8
- 0.5 ml. standard antibody solution, containing 2 antistreptokinase units per ml. (The antibody solution is heated at 56° for 30-45 minutes to destroy the small amount of plasminogen and inhibitor which it contains.)

The mixture of streptokinase and antibody is incubated at 35° for 30 minutes. At the end of this time, the following reagents are added:

- 0.5 ml. of a 0.25% solution of Fraction III
- 0.5 ml. of 0.5% bovine fibrinogen
- 0.1 ml. Hemostatic Globulin, diluted 1:3.

The tubes are incubated for another 30 minutes and the highest dilution of streptokinase which fails to lyse the clot is taken as the end point. A solution of crude or purified streptokinase standardized in this manner should show no significant change in titer over a period of a week or two if the streptokinase concentration is above about 2,000 units per ml. for the crude and above

10,000 units per ml. for the purified. More dilute solutions are not quite so stable, probably because of denaturation occurring in dilute protein solutions. However, in practice, the streptokinase solution is standardized every day or two.

In determining the antistreptokinase titer of serum the serum must be heated at 56° for 30–45 minutes to destroy the plasminogen and inhibitor which it contains. As may be seen in Table II,

TABLE II
Effect of heating serum at 56° C for 30 minutes on serum inhibitor and antistreptokinase

Serum number	Inhibitor equivalent to micrograms trypsin		Antistreptokinase	
	before heating	after heating	before heating	after heating
1	1000	100	128	128
2	500	80	64	64
3	1000	—80*	64	32
4	667	100	64	32
5	667	—80*	128	64
6	667	—80*	16	8
7	1800	120	2560	2560
8	800	48	3200	3200

* Inhibitor equivalent to less than 80 μ g.

heating reduces the inhibitor concentration by approximately 90%, and produces no significant change in the antibody titer. A more valid reason for heating the serum than for the inactivation of inhibitor is to destroy plasminogen, since if this is not done, in the case of sera with a low antibody content, the tubes containing the lowest dilutions of serum will lyse the standard clot because of the activation of the plasminogen in the serum by uncombined streptokinase.

The inactivated serum is titrated for antistreptokinase as follows:

0.5 ml. inactivated serum dilution

0.5 ml. streptokinase solution, diluted in gelatin buffer to contain about 1–2 units.

The tubes are incubated for 30 minutes, and the remainder of the titration carried out as above. The end point is taken as the highest dilution of serum preventing lysis of the clot, and contains antistreptokinase equivalent to the streptokinase in the test.

3. *Serum inhibitor*: A number of methods have been devised in the last few years for the quantitative estimation of serum inhibitor (14, 18, 21–

23). These methods, however, have certain disadvantages for routine use.

Kunitz (13) has shown that crystalline soybean inhibitor undergoes an immediate, stoichiometric, irreversible reaction with trypsin, and that active trypsin can readily be determined by titration of the amount of soybean inhibitor necessary to inhibit the proteolytic activity of the trypsin sample, since the two are equivalent, weight for weight. Schmitz (24) has isolated a small amount of inhibitor from serum which he concluded was probably identical with the pancreatic inhibitor. It has also been shown (14) that serum and pancreatic inhibitor behave in the same way toward trypsin and toward plasmin, although the two inhibitors behave toward trypsin in a quantitatively and perhaps qualitatively different manner than they behave toward plasmin. In view of these facts it was decided to determine the inhibitory power of serum in terms of its ability to inhibit trypsin rather than plasmin. Additional reasons influencing this decision were as follows: (a) crystalline trypsin is readily available and easily standardized, whereas no plasmin preparations of comparable purity are known; (b) trypsin is much more susceptible to the action of inhibitor than is plasmin; and (c) active trypsin can be conveniently stored in a stable solution (in M/400 HCl) while plasminogen and plasmin preparations are very unstable (14, 25, 26).

Standardization of trypsin with soybean inhibitor is carried out as follows:

0.5 ml. freshly prepared dilutions of stock trypsin in borate buffer, pH 7.4–7.6

0.5 ml. soybean inhibitor, usually containing 10 μ g.

1.0 ml. bovine fibrinogen, 0.2% in borate buffer.

The tubes are incubated for 10 minutes at 35°. At the end of this time 0.1 ml. of Hemostatic Globulin, diluted 1:3, is added and the formation of clots noted in 5–10 minutes. The method is based on Ferguson's (27) procedure for the fibrinogenolytic assay of tryptases. The lowest dilution of trypsin in which a clot forms contains trypsin equivalent to the soybean inhibitor in the test. It is necessary to allow the trypsin to act in the absence of Hemostatic Globulin because the Hemostatic Globulin preparations used con-

tain some trypsin inhibitor.⁹ An advantage to this procedure, however, is that the addition of Hemostatic Globulin stops all tryptic activity, so that once the clots are formed, they are stable. Any desired range of trypsin concentration can be titrated by this method by using appropriate concentrations of inhibitor in the test. The sensitivity obtained depends on the interval between successive trypsin dilutions.

The inhibitor concentration of serum is determined as above with the substitution of dilutions of serum for the soybean inhibitor and the substitution of a fixed amount of active trypsin, usually about 10 μ g., for the trypsin dilutions. The tube containing the highest dilution of serum in which a clot forms on addition of thrombin contains serum inhibitor equivalent to the trypsin in the test. Results are expressed as micrograms of active trypsin inhibited by 1 ml. serum.

The accuracy of the fibrinogenolytic test was compared with the method of Kunitz (13) for determining trypsin inhibitor, with results as shown in Table III.

TABLE III

Comparison of methods for serum inhibitor determination
Inhibitor equivalent to micrograms trypsin inhibited per ml. serum

Serum number	Kunitz method	Fibrinogenolytic method
1	520	500
2	490	500
3	590	660

4. *Plasminogen*: Plasminogen determinations of serum are of doubtful accuracy because plasminogen cannot be separated quantitatively from serum, nor can the interfering substances, antistreptokinase and serum inhibitor, be removed or destroyed without loss of plasminogen. However, in samples of sera which are not abnormally high in antibody or inhibitor, the relative concentrations of plasminogen can be determined by activating with streptokinase and determining the least amount of activated serum necessary to lyse a standard clot. The details of the determination are as follows:

0.5 ml. serial dilutions of serum in borate buffer

⁹ As noted above, Hemostatic Globulin is no longer available. Preliminary experiments with the more highly-purified bovine preparation of Parke-Davis indicates that they contain little or no inhibitor.

0.1 ml. streptokinase solution, previously standardized as outlined below

0.4 ml. bovine fibrinogen, 0.5% in borate buffer

0.1 ml. Hemostatic Globulin, diluted 1:3.

The highest dilution of serum lysing the clot in 30 minutes at 35° represents one unit of plasmin, or plasminogen.

It is necessary to activate the plasminogen in the presence of substrate, because, if this is not done, the total protease activity declines rapidly, probably because of the two-fold effect of auto-digestion (25) and combination with serum inhibitor. Activation in the presence of substrate appears to minimize these effects. Since the activation of plasminogen by streptokinase appears to be a catalytic reaction (2, 14, 28-31) it will be most efficient when an excess of streptokinase is used. A large excess of streptokinase would also prevent interference with the reaction by the antistreptokinase contained in variable amounts in practically all human sera. When attempts are made to use maximal amounts of crude streptokinase to activate serum plasminogen, an inhibitory effect is noted. This inhibitory effect of undiluted, concentrated crude streptokinase is due, probably, to the presence in crude streptokinase preparations of a protease inhibitor (14) which decreases in amount as streptokinase is purified. When purified streptokinase is used to activate a serum, maximum protease activity is obtained with the highest concentration of streptokinase, as shown in Table IV.

TABLE IV

Plasminogen activity of serum activated with various concentrations of purified streptokinase

Purified streptokinase dilution	Highest dilution of serum causing lysis in 30 minutes
undiluted	5120
1:10	5120
1:100	1280
1:1000	160

In addition to the absence of an inhibitory effect of high streptokinase concentration when using purified material, it can be seen from the data in Table V that the activity obtained is considerably higher than with the crude material. It is obvious, therefore, that activation with purified material is the method of choice. Unfortunately, it has not been possible in the past to obtain sufficient purified material for all requirements of the stud-

ies involved in these reports. Therefore, protease determinations were made with selected batches of crude streptokinase, titrated to determine those lots and dilutions which would give the highest

are presented the results obtained by using the methods described above.

TABLE V

Relation between maximum plasmin activity developed in serum by crude and purified streptokinase

Serum	Maximum plasmin activity	
	Crude streptokinase	Purified streptokinase
L (6-19-48)	1280	3200
L (7-16-48)	640	1600
S	320	1280
B	160	1280
F	320	2560

values with normal sera. Such lots were set aside and used in all the protease determinations reported in the present studies. The results obtained, while not representing the maximal values, are useful as a measure of the relative plasminogen content of sera. The validity of this assumption is borne out by the observation (Table V) that a direct relation exists between the maximum amount of protease activity obtained on the activation of serum with crude and with purified streptokinase.

5. *Desoxyribonuclease*: The determination is carried out as described by McCarty (7), except that drop in relative viscosity is measured over a 10-minute instead of a 20-minute period of incubation. A unit is defined as the amount of enzyme necessary to cause a drop of 1 viscosity unit in 10 minutes at 30° in a reaction mixture consisting of 2.4 ml. of desoxyribose nucleic acid and 0.1 ml. enzyme solution. The substrate consisted of a 0.15-0.2% solution of thymus desoxyribose nucleic acid in veronal buffer. The nucleic acid was prepared as described by Mirsky (32). Difficulty was encountered with the nucleic acid solutions in that a spontaneous drop in viscosity was noted. This effect could be eliminated by heating the nucleic acid solution to 56° for about an hour. The heated solution, when used in the reaction mixture described above, had an initial relative viscosity of 4-5 units.

RESULTS

1. *Antistreptokinase*: Evidence is abundant that an antibody to streptokinase appears following streptococcal infection (18, 33-36). In Table VI

TABLE VI
Antistreptokinase levels in various sera

Serum	No. samples	Antistreptokinase units	
		Mean	Range
Normal	14	36	4-80
Infants (under 1 year)	9	8	0-29
Rheumatic fever (children)	8	1302	100-4200
Streptococcal pharyngitis	3	92	80-115
Tuberculous pleurisy	22	87	2-400
Dog	3	102	72-156
Monkey	1	66	
Cow (pool)		8	
Horse (pool)		4	
Chicken	1	32	

Direct evidence that antistreptokinase is produced in humans following injection of streptokinase has been obtained in the present series of studies and is reported more fully in the accompanying paper (1). In Table VII are shown the antibody titrations in the serum of two patients following the intrapleural injection of purified streptokinase. The antibody responses seen in the two patients reported in Table VII are perhaps

TABLE VII

Rise in antistreptokinase following intrapleural injection of purified streptokinase

Patient	Date	ASK units
M. H. (female)	6/8 (200,000 SK injected intrapleurally)	20
	6/11	80
	6/17	5120
	6/22	over 10,000
C. L. (male)	5/13 (50,000 units Sk injected intrapleurally)	40
	5/17 (100,000 units SK)	—
	5/20	5120
	6/9	over 10,000
	6/22	over 10,000
	7/2	5120

somewhat more rapid than is usual as a primary response to the injection of an antigen. The rapidity of the response, however, is probably due to the fact that the response is an anamnestic one, since it will be noted that both patients had a titer before injection of streptokinase. In fact, all human sera tested have had some antibody, even

with no known history of streptococcal infection.

2. *Serum inhibitor*: The values obtained for a variety of sera are presented in Table VIII. Results are expressed as micrograms of active trypsin inhibited by 1 ml. of serum.

TABLE VIII

Serum inhibitor levels of serum from man and animals

Serum	No. of samples	Serum inhibitor as micrograms trypsin inhibited per ml.	
		Mean	Range
Normal adult	16	655	100-1200
Infants (under 1)	9	613	420-1024
Rheumatic children	5	760	570-900
Pulmonary tuberculosis	7	1035	660-1500
Carcinoma	4	883	600-1333
Leukemia	5	1096	842-1350
Chicken	1	264	
Rabbit	1	400	
Horse (pool)	2	765	670-860
Dog	1	365	
Guinea pig	2	2125	1750-2500

The data presented are obviously too scanty to warrant the drawing of conclusions. However, the finding that the value is elevated in chronic wasting diseases such as tuberculosis and malignancies is in accord with many earlier observations on serum inhibitor (see Grob [21] for references).

The values for animal sera tested lie within the range of normal human values, with the exception of guinea pigs, which, at least in the few specimens tested, have an abnormally high value.

TABLE IX

Levels of plasminogen in various sera

Serum	No. of specimens	Units of plasminogen	
		Mean	Range
Normal adult	8	572	160-1280
Rheumatic children	3	960	320-1280
Tuberculosis	6	4053	1280-5120
Carcinoma	1	2560	
Non-rheum. H. D.	5	576	320-640
Chicken	1	Less than 10 units	
Monkey	2	Less than 10 units	
Dog	3	Less than 10 units	
Cattle (pool)	1	Less than 10 units	

Of interest are the somewhat higher values for rheumatic children. If these differences are significant, their relation to the higher plasminogen values obtained for rheumatics (Table IX) is of

importance, suggesting the possibility that the inhibitor might serve a role in relation to plasminogen. However, when one considers the inhibitor and plasminogen values obtained with animal sera, no relationship appears to exist, since the inhibitor level is high while the amount of plasminogen activatable by streptokinase is negligible.

3. *Plasminogen*: The deficiencies of the method for plasminogen determination have been discussed above. The results obtained are presented in Table IX.

In spite of the deficiencies of the method, the results obtained with normal sera have been reasonably uniform, the great majority of them showing levels of 320 or 640 units. Retitration of a single serum, or titrations of successive bleedings of the same individual show a variation of no more than one tube in the series. The higher values obtained with certain of the patients under investigation (*e.g.*, tuberculous and carcinomatous) probably represent an increase over normal, even though the method of titration does not give maximal values for any serum. If these values are artifacts and do not represent an increase of total plasminogen over normal, it is difficult to account for the results, since the two substances in serum known to influence the reaction, serum inhibitor and antistreptokinase, would both tend to decrease, rather than increase the plasminogen values.

SUMMARY

A method for purification of streptokinase describes conditions for obtaining high yields from bacterial cultures. Protamine is used to remove impurities and streptokinase is separated from desoxyribonuclease with cold ethanol at 20% concentration. The desoxyribonuclease is precipitated with 40-50% cold ethanol. The yield of streptokinase is about 25% and the product so obtained has been used in clinical studies.

Methods are briefly described for the quantitative estimation of streptokinase, antistreptokinase, serum inhibitor, serum plasminogen, and desoxyribonuclease. These methods were employed to make a survey study of various sera. There is a marked rise in antistreptokinase activity following intrapleural injection of streptokinase in human beings. Serum from the normal

adult contains more antistreptokinase than serum from the infant and the level is strikingly high in rheumatic fever. In various species the concentration decreases in the following order: dog, monkey, man, chicken, cow and horse. Serum inhibitor levels show elevations in wasting diseases, and in the animals studied the guinea pig has an exceptionally high inhibitor titer. The method for plasminogen determination presents difficulties but it appears that there may be a marked increase in patients with tuberculosis. The concentration in chicken, monkey, dog, and cattle appears to be low.

Serum can be heated at 56° C for 30 minutes to destroy serum inhibitor but antistreptokinase is not destroyed.

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THE EFFECT IN PATIENTS OF STREPTOCOCCAL FIBRINOLYSIN (STREPTOKINASE) AND STREPTOCOCCAL DESOXYRIBONUCLEASE ON FIBRINOUS, PURULENT, AND SANGUINOUS PLEURAL EXUDATIONS¹

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The results described in this article were obtained by the injection of concentrated and partially purified preparations derived from broth cultures of hemolytic streptococci into the pleural cavity of selected patients who were suffering from different types of diseases that gave rise to pleural exudations. The possibility has been explored of utilizing two of the defined properties elaborated by hemolytic streptococci that have the unique capacity of causing rapid lysis of the solid elements (fibrin and nucleoprotein) that are significant parts of exudates. In a companion article, Christensen (1) has described additional steps in the purification of the materials employed and has given quantitative methods of measurement of various increments concerned in the fibrinolytic system, including trypsin inhibitor and specific antibodies, which have been employed in this study.

In the study of patients it was first necessary to: (a) develop the investigation within non-toxic but effective ranges of doses of the material employed, and (b) determine whether or not the enzymatic activities of the streptococcal products were effectively operative when introduced directly into the site of the disease in the patients. This article is essentially limited to findings that are pertinent to the two aspects of the study mentioned above.

A brief description of the two streptococcal products that have been the subject of the investigation is as follows:

I. *Streptococcal fibrinolysin* (2) (*Strepokinase*)

This has been found to be elaborated characteristically by strains of hemolytic streptococci (Group A) causing acute infections in patients. It is also produced by some strains of strepto-

coccal groups C and G (3). The product is abundantly excreted into the culture medium in which the organisms are grown and is readily obtainable free from the bacterial cells in sterile filtrates.

The fibrinolytic action, in tests conducted under optimal laboratory conditions, is unusually rapid in action on the fibrin coagulum of normal human blood, requiring only a few minutes when whole plasma is employed as a source of fibrin, and an even shorter time when preparations of fibrinogen and thrombin of human origin are used. The ultimate proteolytic nature of the fibrinolysis has been evident in the liberation of breakdown products of protein digestion (4).

Another interesting feature of the phenomenon is the high degree of activity of the streptococcal lytic system in the presence of normal human blood as contrasted with its inaction or delayed effect when tests are conducted with the blood of various animal species with the exception of monkeys (3). Although studies of factors involved in streptococcal fibrinolysis have indicated a basis for the differences in the behavior of blood from different animals, nevertheless the existence of the difference has rendered impossible most experimental studies, *in vivo*, on animals and has limited them to observations conducted in patients, such as those initially described in this article.

The mechanism of the reaction has been a subject of study by various investigators.

In an earlier report (2) it was noted that fibrin formed from mixtures of either human fibrinogen and rabbit thrombin, or of rabbit fibrinogen and human thrombin were susceptible to streptococcal lysis, whereas the fibrin derived from rabbit fibrinogen plus rabbit thrombin was unaffected. Milstone (5) demonstrated that an increment of human serum present in the euglobulin fraction,

¹ This study was supported by a grant from the National Institute of Health, United States Public Health Service.

was necessary for the reaction to occur and that if preparations of human fibrinogen and thrombin were highly purified no lysis would occur when the streptococcal product was added. Furthermore, if this human globulin fraction was added to fibrin made up of rabbit components, lysis occurred. He referred to the euglobulin constituent of human blood as "lysing factor" and suggested that streptococcal fibrinolysis was mediated by the same system as that involved in other examples of fibrinolysis.

Christensen (4), and Christensen and MacLeod (6) have presented results concerning the mechanism, and have concluded that the streptococcal product to which they have given the name streptokinase acts as a catalyst or kinase in causing the transformation of a zymogen of normal human blood, designated by them plasminogen, into a proteolytic enzyme, designated plasmin.

Kaplan (7) has made extensive studies on several aspects of the mechanism of the streptococcal fibrinolysin-serum protease system. These studies have in part dealt with the dissimilarity of serum protease and trypsin and also with the inhibition of streptococcal fibrinolysis by antiprotease. The findings are predicated upon the kinase nature of the action of the streptococcal product and the identity of the protease of serum as the lysing principle. Ratnoff (8), studying a proteolytic enzyme in human plasma, gave special attention to the fibrinolytic system activated by the streptococcal product and obtained data indicating that the activation of plasma proteolytic enzyme by streptococcal fibrinolysin behaved as if it involved a stoichiometric reaction. This finding implies that the streptococcal product may take an active part in the lytic system rather than exert only a catalytic effect.

Without developing the discussion of the mechanism, it is sufficient to state that the objective demonstration of changes in constituents of pleural exudates that have been effected by the streptococcal fibrin-dissolving system, acting within the pleural cavity of patients, constitutes definitive results around which this report centers. The conditions, unavoidably inherent in the several factors that may be simultaneously operative in association with the processes of disease within patients, makes it impossible at the present time to analyze and identify the mecha-

nism of fibrinolysis that may be responsible for the end results that we have observed.

Nomenclature. The fibrinolytic principle derived from hemolytic streptococci is referred to in this article as *streptokinase*. The kinase type of action of the streptococcal product has been consistently observed by several investigators. However, whether or not its complete activity is solely that of kinase remains to be determined.

The term *fibrin-lysing system* has been employed as an objectively descriptive expression which avoids the different recommendations of various investigators (6, 9-11). The *activatable fibrin-lysing system* may be closely identified with the plasminogen of Christensen and MacLeod (6), and the *active fibrin-lysing system* with their term plasmin.

II. *Streptococcal desoxyribonuclease*

In recent articles the occurrence of desoxyribonucleoprotein in significant amounts in purulent empyematous fluids was reported (12) and its depolymerization by a desoxyribosenuclease found to be present in the concentrated streptococcal filtrates was also described (13).

The solid sediments of the samples of pus were found to contain as much as 30% to 70% nucleoprotein. Since the physical characteristics of desoxyribose nucleoprotein are fibrous and gelatinous, the similarity of its appearance to that of fibrin became a matter of importance in identifying the dual enzymatic systems with which we were dealing; namely, fibrin substrate in the fibrinolytic system, and nucleoprotein substrate acted upon by nuclease. In each instance rapid liquefaction of the respective solid substrate occurred.

Each of the substrates mentioned has been identified in varying amounts in different types of pleural exudation. For example, empyematous pus of bacterial origin is high in its content of nucleoprotein, which is presumably derived from the leucocytes, while the constituent most conspicuously present in hemothorax is fibrin. Fibrinous pleural exudates such as may be encountered in association with pneumonia, pulmonary neoplasm, and other diseases, or the effusions of tuberculous pleurisy have been studied chiefly for the fibrinolytic effect, although from

observations on preparations of the exudates stained by the Feulgen method (12), it is evident that varying amounts of nucleoprotein are present.

*Streptococcal concentrates*²

The preparations employed in this study have been prepared by Christensen according to the method of partially purifying the concentrated filtrates of hemolytic streptococci previously described and further elaborated in the accompanying article (1, 14). A strain of hemolytic streptococcus (H46A) belonging to Lancefield Group C has been employed. This strain is potent in its production of both streptokinase and desoxyribose nuclease (13) and most of the lots of concentrated filtrate used in this study have possessed both properties. In addition it seemed reasonable to assume that since the strain (a group C) was not of patient origin its products might presumably be least likely to contain other noxious, but as yet unidentified, increments associated with acute infections in man and would, therefore, be more desirable for preparing material to be used in patients than would a Group A fibrinolytic strain. All the partially purified and concentrated preparations, with one exception, that were introduced into patients, contained both streptokinase and nuclease. In an additional preparation used in a single patient with hemothorax, the content of nuclease was reduced substantially by Christensen (1) but the streptokinase activity remained unimpaired.

The dosage of streptokinase has been based upon a *unit*, developed and described in the accompanying article by Christensen (1). The dosages per patient are given in the individual protocols. In general they ranged from 20,000 to 400,000 units. The concentration per cubic centimeter ranged from 2,000 to 40,000 units. The total dose was usually contained in 10 cc. of normal saline solution.

The desoxyribonuclease content of the various preparations of streptococcal concentrates has not yet been elaborated so extensively as that of streptokinase. Christensen (1) has suggested a unit basis which is at present being employed. In general the concentrates contained a ratio of approximately 6,000 units of nuclease to 15,000 units of streptokinase. The nuclease was of special significance in the cases of empyema, and the dosage is given in the description of the results obtained in that group.

Toxicity of streptococcal concentrates. Among the initial studies (2) concerned with the characterization of the streptococcal fibrinolytic phenomenon, preliminary observations (unpublished) were made with respect to the skin reactivity of active filtrates derived from strains of fibrinolytic streptococci. Following intracutaneous injections, areas of local erythema developed that simulated the type of a positive reaction elicited by the erythrogenic scarlatinal toxin. However, by comparative tests in

which both scarlatinal toxin and fibrinolytic filtrates were injected into Dick positive and Dick negative individuals, no correlation was detected. From the limited observations, it seemed reasonable to assume that the primary toxicity of the fibrinolytic filtrate was due either to the fibrinolytic principle itself or to some contaminating product other than the established erythrogenic toxin of hemolytic streptococcal origin.

When the recent concentrates prepared by Christensen (1) in the process of purifying the streptococcal fibrinolytic were being considered for introduction into patients, intradermal tests were performed. Although the concentration of streptokinase was increased many hundred-fold over the preparations of earlier use, the degree and frequency of erythematous reactions were found not to be any greater than those previously encountered nor to be related to the concentration.

Samples of the concentrates were then injected intramuscularly. No reactions occurred that were detectable by the development of local redness, tenderness or swelling, or as a generalized pyrogenic effect. When increasingly large concentrations failed to produce untoward effects intramuscularly, small amounts were introduced intrapleurally into patients with empyema, inflammatory pleural effusions, or hydrothorax.

Without describing the details of the progressive observations following intrapleural injections it may be stated that as the procedures of purification and concentration conducted by Christensen developed, the evidences of toxicity have diminished in relationship to amount injected. The studies have, therefore, up to the present time indicated that the primary toxicity of the fibrinolytic principle or the accompanying desoxyribonuclease is not great and that the transient pyrogenic reaction may be due particularly to contaminating substances still present in even the purest preparations now available.

The manifestations of toxicity when they occurred have consisted of a pyrogenic reaction beginning *approximately* six to eight hours after the injection, reaching its peak of a rise of one to four degrees at 24 hours after injection and decreasing gradually over the next 24 to 72 hours to the pre-injection level which in some instances was a normal temperature and in others was elevated prior to the injection due to the underlying disease. General malaise accompanied the febrile period. Nausea and gastro-intestinal discomfort were noted in some patients. Local pain, although never severe, was present in some instances, and not in others.

An outpouring of polymorphonuclear leucocytes occurred which was demonstrable 24 hours after the injection and decreased to the pre-injection level within the next two to ten days. The increase in the leucocyte counts ranged from 1,000 to 25,000 cells per cubic centimeter.

The general pyrogenic reaction together with individual symptoms as mentioned, and the local outpouring of cellular constituents did not follow any uniform or correlated pattern, since all of the manifestations were not present to the same degree and some of them were entirely absent. In several instances in which two injec-

² Lederle and Co. have cooperated by growing large volumes of culture and supplying the filtrate in its initial concentrated form.

tions were given, the first may have caused a reaction while the second elicited no toxic response, or the first was silent and the second evoked some reaction. Furthermore, the occurrence of reaction was not clearly related to dosage of concentrate.

In this study a total of 34 injections was given to 23 patients. Utilizing a rise of 1° F in fever as an indication of a reaction—and irrespective of whether or not the patient was febrile before the injection—on 17 (50%) occasions a pyrogenic response was noted.

The degree of reaction appeared to be most directly related to the nature of the patient's disease. In instances where considerable thickening of the pleura was present or other circumstances that may have impaired the absorbing surface, minimum or no toxic reactions occurred. In other cases where the contrary situation of a thin pleura or presumably a relatively freely acting absorbing surface was present, the reactions as described were more consistently noted.

Other factors that may participate in the reaction in a manner as yet undetermined are the breakdown products derived on the one hand from the fibrinogen and fibrin system and, on the other hand, from the nucleoprotein system through the action of nuclease. No specific information on this phase of the subject is available except the fact that the toxic reaction, whatever its exact cause may be, was a transient one of the classical brief pyrogenic type without any residual effects. No indication of chronic alterations of an untoward nature have been evident. Because of the implications of hemolytic streptococci in relation to rheumatic fever and acute hemorrhagic nephritis, detailed examinations for their possible presence have been frequently made with uniformly negative findings.

It has been of special importance to make preliminary tests with each new lot of purified concentrates made available for use in patients by injecting a small amount intrapleurally and noting the degree of reaction. When the reaction appeared to be relatively greater than would be expected from the small dose, the lot was not further employed in the study of patients. This precautionary procedure of selecting individual preparations for intrapleural use has been regularly adhered to, and, as a result, no contraindications for the development of the study have been encountered.

PROCEDURE

Observations on Patients. In the study of patients numerous quantitative determinations have been made on samples of exudate obtained prior to the injection of streptococcal concentrates and at stated intervals thereafter, usually one hour, 24, 48, 72 hours, etc., according to the nature of the case and the findings.

The tests and procedures employed have included the following: fibrinogen, N.P.N., total protein, formal titration, amount of sediment, viscosity, pH, volume of pleural exudate, free streptokinase, activatable fibrin-lysing system, active fibrin-lysing system, trypsin inhibitor, and specific anti-fibrinolytic antibody (anti-streptokinase).

Cytological studies, including total and differential counts, determination of motility and viability of cells and the appearance of Feulgen-stained preparations for the purpose of identifying intra- and extracellular deoxyribose nucleoprotein have also been made. These results will be referred to briefly in this article and be reported in detail in a subsequent communication.

The technical procedures in each instance have been as follows:

Streptokinase titre, activatable fibrin-lysing system (equivalent to plasminogen), trypsin inhibitor, and anti-streptokinase titre were determined on chest fluid according to the methods described by Christensen (1). *Active fibrin-lysing system (equivalent to plasmin)* was determined as follows: To 0.1 cc. samples of chest fluid, brought to neutral pH, were added 0.5 cc. of 0.25% Bovine Fibrinogen (Armour) in 0.01 M saline phosphate buffer (pH 7.4), and 0.5 cc. of 0.25% Human Plasma Fraction III (Cohn) in saline phosphate buffer. One tenth ml. of 1/3 dilution of Lederle Hemostatic Globulin (or 1/500 dilution of Parke Davis Bovine thrombin) in saline phosphate buffer was then rapidly added. The tubes were incubated in a water bath at 37°. One tenth cc. of saline instead of chest fluid was used for controls. The time of complete lysis of the fibrin clot was noted. Since the lysis of fibrin in the absence of extra increments of streptokinase was relatively slow and, therefore, could not be expressed in the same unitage scale, the results were expressed as 0 — + + + + as follows:

lysis up to one hour + + + +
lysis one to six hours + + +
lysis six to 24 hours + +
lysis 24 to 48 hours +
lysis 48 to 72 hours but no lysis in controls ±
No lysis 0

Estimations of the degree of activity of the fibrin-lysing system with respect to time have proved important since the period required for lysis, *in vitro*, reflected to some degree the activity of the process, *in vivo*, which was significant with respect to changes in the exudates that continued for several days after the initial injection.

Amount of sediment: Wintrobe hematocrit tubes were filled to the mark with a heparinized sample of chest fluid and centrifuged at 2,500 rpm for one hour. The results are expressed as per cent.

pH was determined on separately collected heparinized samples by glass electrode after chest fluid was allowed to equilibrate with room temperature. A previously determined temperature correction for the instrument used was applied, so that the results are expressed as pH at 37° C.

Viscosity: The viscosity of thin fluids was determined on heparinized samples in an Ostwald viscosimeter at 37° C. The viscosity of thick purulent fluids was determined with an LV model Brookfield electric viscometer at 37° C.

Total protein and N.P.N. were done on the supernatant of centrifuged oxalated specimens of chest fluid. The total protein was determined by Kjeldahl digestion and nesslerization. The N.P.N. was determined by digestion and nesslerization of a trichloroacetic acid filtrate. The results are expressed as milligrams per cent for N.P.N., and grams per cent for total protein.

Fibrinogen was determined on the supernatant of centrifuged oxalated specimens. Five-cc. samples were diluted to 30 cc. with normal saline, to which were added 1 cc. of 2.5% CaCl₂ solution and 1 cc. of a 1/100 dilution in normal saline of Parke, Davis Bovine Thrombin. The tubes were then placed in the refrigerator overnight. The clots were removed with a stirring rod, washed with distilled water, and the nitrogen determined by digestion and nesslerization. The results are expressed as milligrams per cent.

Formol titration: Ten-cc. samples of heparinized fluid were brought to pH 7.0 by addition of either 0.1 N NaOH or 0.1 N HCl. Five cc. of 40% formaldehyde were added, and the mixture titrated with standard 0.1 N NaOH by glass electrode to pH 8.6. The volume of alkali necessary to bring 5 cc. of 40% formaldehyde to pH 8.6 was subtracted from the titration. The results are expressed as cubic centimeters of 0.1 N NaOH per 100 cc. of chest fluid.

Volume of pleural fluid: Since information concerning the volume of pleural effusions was desirable, the following method was developed. The fluid volume was determined in pleural transudates and non-purulent exudates by injecting known quantities of T-1824 and sampling the pleural fluid from 15 to 60 minutes thereafter. This method is not applicable to purulent empyemas where a significant portion of the dye is bound to the sediment of the fluids and probably to the shaggy pleural surface, but quantitative determinations on non-purulent pleural effusions have yielded satisfactory results.

RESULTS

Twenty-three patients have been studied following the injection of amounts of streptococcal concentrates sufficiently large to bring about definitive and measurable biochemical and biophysical changes in the area of exudation at the site of the injection. Although the general plan of study has been the same in all instances the presentation of the results has been arranged according to a grouping of the cases based on the types of exudates which were present in the pleural cavities of the patients. The changes that have been effected by the concentrates have been found to bear a relationship to the character of the exudates.

The three major groups were: acute fibrinous

pleurisy, bacterial empyema, and hemothorax.³
Group I. Acute fibrinous pleurisy, 13 patients.

Five cases of tuberculous pleurisy with effusion.
Six cases of primary or metastatic malignant pulmonary neoplasm with pleural effusion.
Two cases of congestive heart failure with pleural effusion.

Figure 1 contains data derived from serial determinations of fibrinogen and nonprotein nitrogen made on seven patients before and after the introduction of streptococcal concentrate. The dosages, given in the figure, ranged from 20,000 to 200,000 units of streptokinase, and approximately 20 to 200 units/cc. chest fluid. Three of the patients received a second injection.

The fibrinogen represents the chief parent protein (plus an undetermined amount of solid fibrin) that was acted upon by the fibrin-lysing system, and the N.P.N. represents the end-product of the local proteolysis.

The findings in each of the seven patients demonstrate the rapidity of the beginning decrease in measurable fibrinogen and increase in N.P.N., the samples taken as early as one hour after the injection revealing changes in the values of each. In patient W. H., for example, the immediate drop in fibrinogen was from 41 to 20 mgm.%, and in patient J. G., from 108 to 26 mgm.%. The concomitant rise in N.P.N. in patient W. H. was from 45 to 52 mgm.%, and in patient J. G., from 31 to 41 mgm.%.

In five of the seven patients of the charted group, a further drop in fibrinogen was noted in the specimen obtained 24 hours after the injection, and in each of the seven the N.P.N. either continued to rise or maintained a higher level than that of the pre-injection figures.

Three of the patients received a second injection of streptococcal concentrate; patient C. L. received 100,000 units of streptokinase four days later, patient E. H. received 40,000 units of streptokinase three days later, and patient J. G. received 200,000 units of streptokinase three days later. In each case there was a further abrupt

³ The authors wish to acknowledge the cooperation and many helpful suggestions which they have received from the Attending and Resident Medical and Surgical Staffs of the Chest Service of Bellevue Hospital, Dr. J. Burns Amberson, Director.

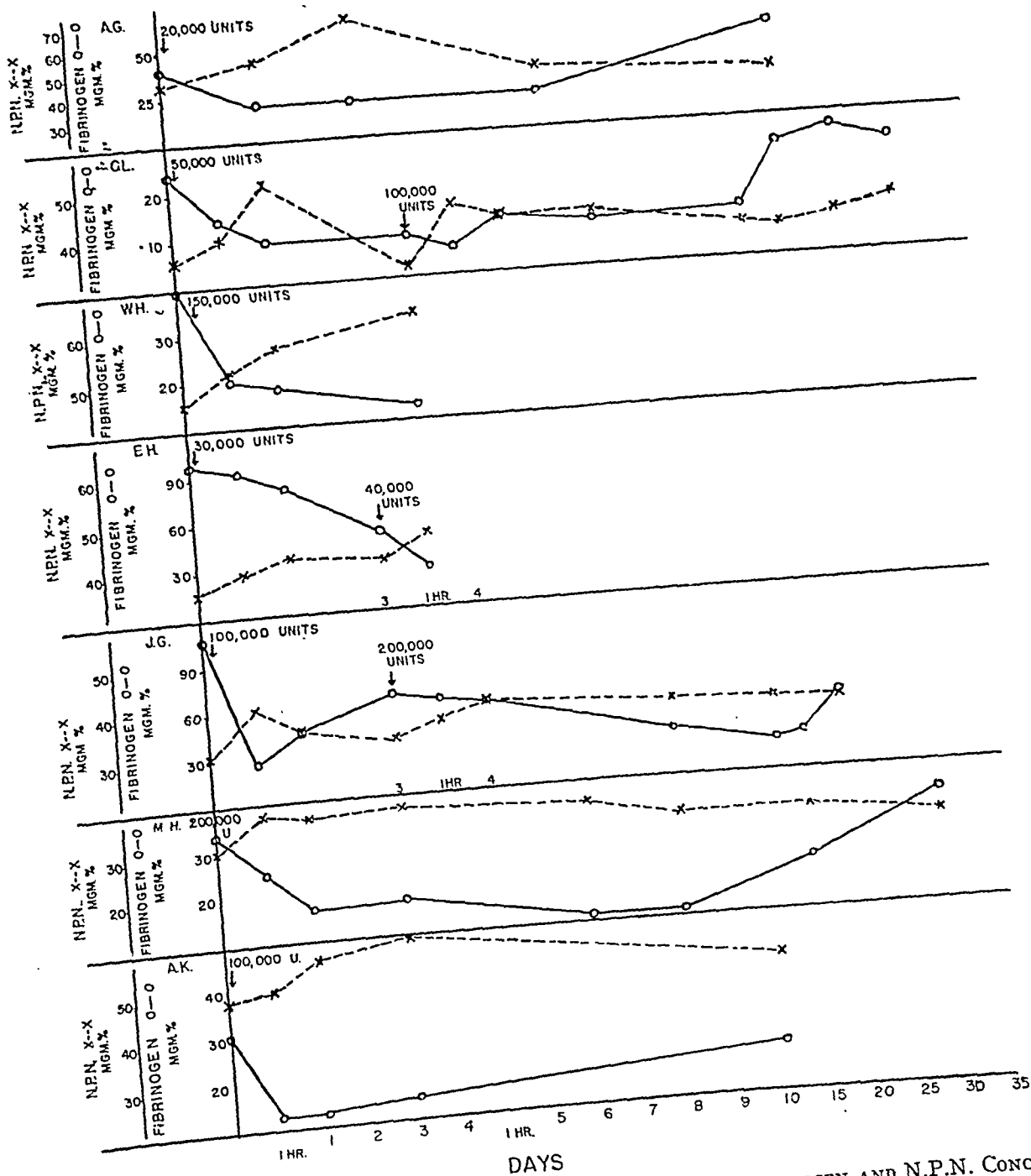


FIG. 1. THE EFFECT OF INTRAPLEURALLY INJECTED STREPTOKINASE ON THE FIBRINOGEN AND N.P.N. CONCENTRATIONS OF THE CHEST FLUID OF SEVEN PATIENTS WITH FIBRINOUS PLEURAL EFFUSIONS. The crosses represent N.P.N. (mgm.%), and the circles, fibrinogen (mgm.%).

decrease in fibrinogen and rise in N.P.N. following the second injection.

In five cases in which the fibrinogen content was followed for long periods of time by examining samples of pleural fluid obtained at repeated intervals, the levels of fibrinogen were found to return to the control value only after ten days or one month. Why the fibrinogen remained low for this period of time is not readily apparent.

Continuing lytic action during this period was not evident by the measurements of the active fibrin-lysing system employed. Furthermore, the fibrinogen levels remained low despite the appearance of antistreptokinase, and was independent of the reappearance of additional increments of the activatable fibrin-lysing system through further exudation of serum into the pleural area. It may represent the time necessary for the exudation

additional fibrinogen to occur into the area of pleurisy, or slow proteolysis may have continued but at a rate not determinable by our present methods.

There were in the patients several uncontrollable factors that altered the serial findings as they extended over several days. For example, when the patient's disease was in an acute active state,

there was a continuing increase in the effusion together with the inflammatory constituents. When the disease was stationary or subsiding, the contents of the pleural cavity were not substantially altered during the period of observation. In addition, the variable degree of local irritation caused either by the injection material itself or the breakdown products of proteolysis contributed

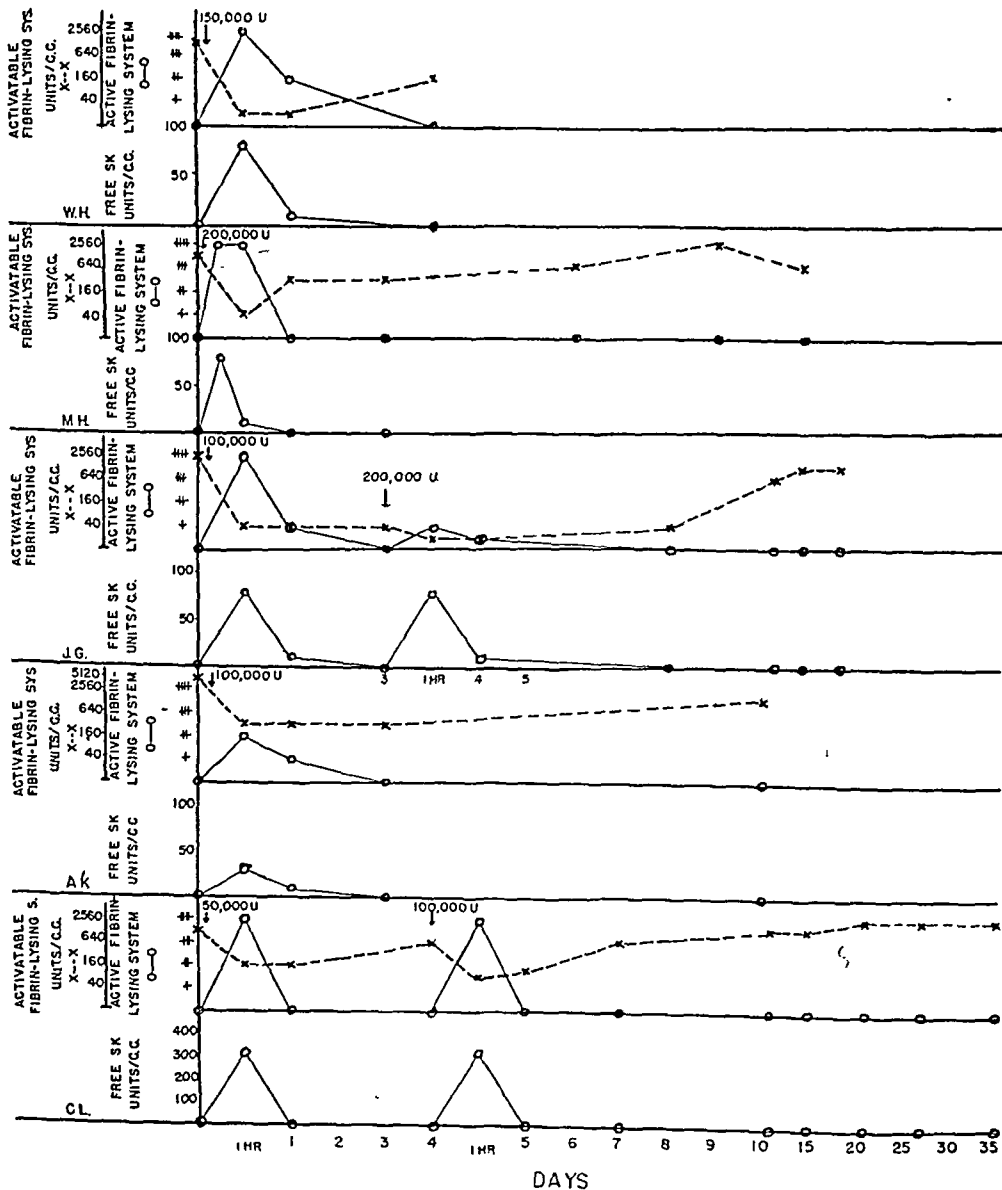


FIG. 2. THE EFFECT OF INTRAPLEURALLY INJECTED STREPTOKINASE ON THE FREE STREPTOKINASE, ACTIVATABLE AND ACTIVE FIBRIN-LYSING SYSTEMS OF THE CHEST FLUID OF FIVE PATIENTS WITH FIBRINOUS EXUDATES

In the graphs for each patient, in the top sections the crosses represent the activatable fibrin-lysing system (units/cc.) and the circles the active fibrin-lysing system; in the lower sections, the circles represent free streptokinase (units/cc.).

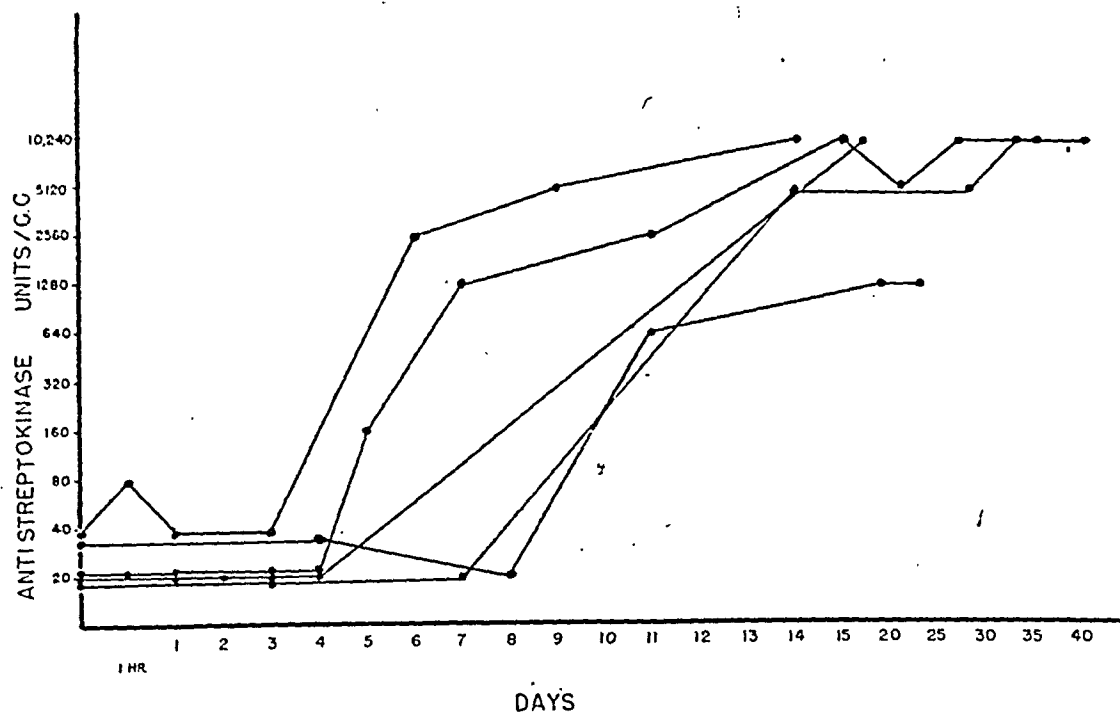


FIG. 3. THE ANTISTREPTOKINASE TITRE IN PLEURAL EFFUSIONS FOLLOWING INTRAPLEURAL ADMINISTRATION OF STREPTOKINASE IN FIVE PATIENTS WITH FIBRINOUS PLEURAL EXUDATES
Streptokinase was injected at zero time.

an additional increment of exudate. Furthermore, the rate of absorption of the newly formed non-protein nitrogen proceeded at variable rates. Consequently, the figures obtained by analysis of the exudate were only relative with respect to total proteolysis.

In spite, however, of the variables outlined above, the intrapleural degradation of fibrinogen in significant amounts following each injection of the streptococcal concentrates has uniformly occurred in each of the patients in this series, as well as in those of the other groups. The extent to which solid preformed fibrin underwent lysis was not measurable but its breakdown undoubtedly contributed to the N.P.N. that was liberated.

In some of the patients, estimations of blood N.P.N. were done but no significant rise above normal levels was noted, the rate of absorption from the pleural area being insufficient to raise the content of the general circulation.

The data contained in Figure 2 concern three elements involved in the streptococcal fibrin-lysing system, namely: free streptokinase, and both the activatable and the active fibrin-lysing system. They are recorded for five patients of the acute fibrinous pleurisy group. Similar findings were

obtained from most of the patients studied of all the groups.

Prior to injection the amount of activatable fibrin-lysing system present in the pleural exudate was approximately the same as that found in normal serum but within one hour after the injection of streptokinase striking changes occurred in the elements of the fibrin-lysing system of the pleural exudate. When streptokinase was introduced, the activatable elements of the lytic system were replaced by active increments which were demonstrable immediately following injection but progressively disappeared during the next 24 to 48 hours. This implies that once the system assumes the active form it is subsequently destroyed, or disappears through combination with substrate. Its replenishment must await, through further exudation from the general circulation into the local area, the addition of new components of the activatable system which were rapidly depleted following the introduction of streptokinase. The rate at which the replenishment occurred has depended upon factors that control the reformation of exudate. In the series of cases used in this study, it has been demonstrable within a few days. These findings indi-

J.G. - FIBRINOUS EXUDATE

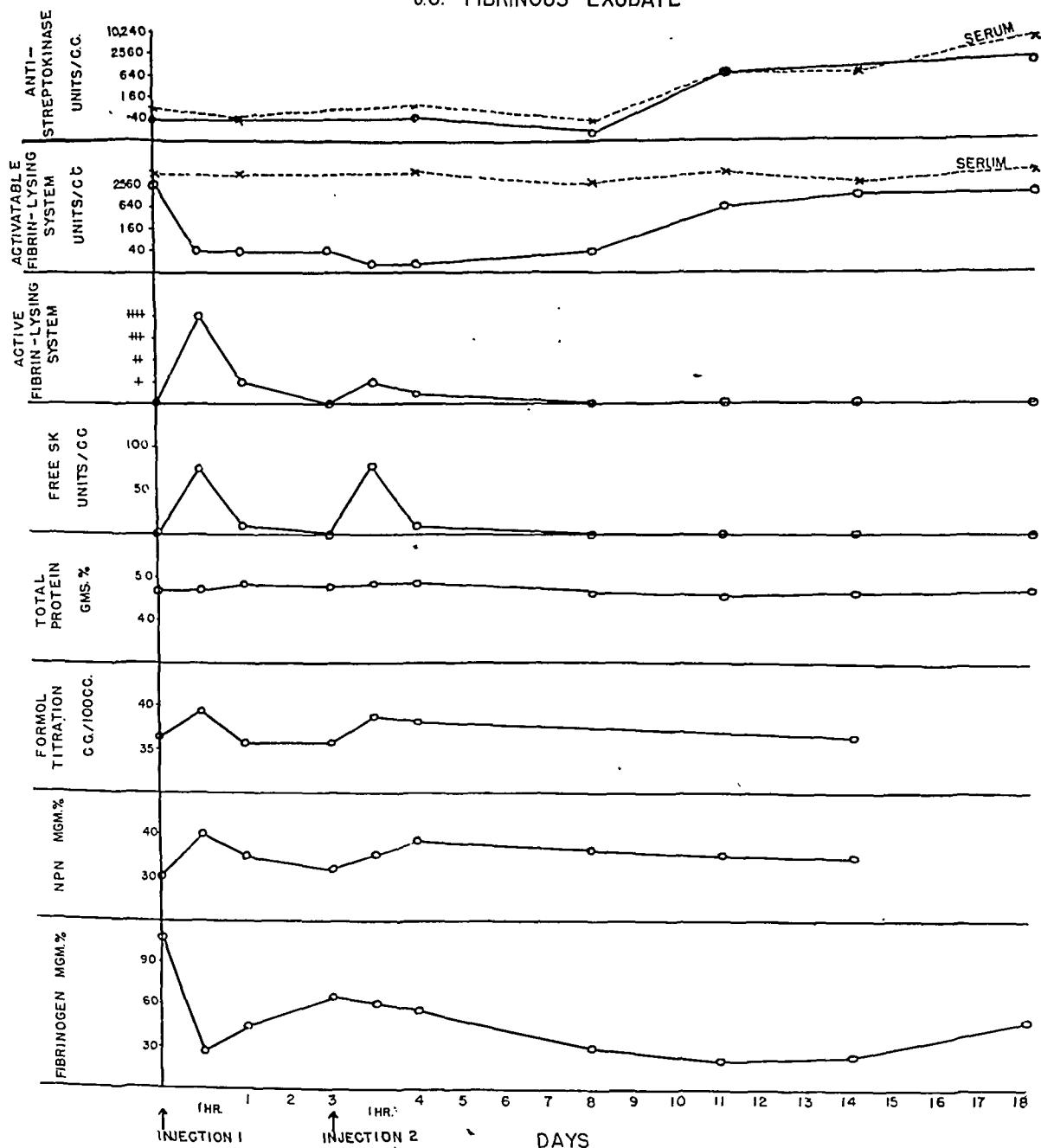


FIG. 4. THE EFFECT OF TWO INTRAPLEURAL INJECTIONS OF STREPTOCOCCAL CONCENTRATE IN A CASE OF TUBERCULOUS FIBRINOUS EXUDATE ON THE FIBRINOGEN, N.P.N., FORMOL TITRATION, TOTAL PROTEIN, FREE STREPTOKINASE, ACTIVE AND ACTIVATABLE FIBRIN-LYSING SYSTEMS, AND THE ANTISTREPTOKINASE TITRE

100,000 units of streptokinase were given in injection 1, and 200,000 units in injection 2.

cate the self-limiting nature of the activity following a single injection of streptokinase.

In our observations trypsin inhibitor has not changed after streptokinase injections and the results indicate that this inhibitor plays little

part under these circumstances in the inactivation of the system.

An excess of free streptokinase was found to be present one hour after the injection but was not demonstrable 24 to 48 hours later. The extent

to which it may be dissipated by combining directly with substrate, or by inactivation by some inhibitor, or by neutralization with specific antibody is not at present determined. Also suggested by the data in Figure 2 is the finding of active fibrin-lysing elements *only* in the presence of free streptokinase. This observation may have a bearing on the mechanism of the phenomenon as it occurs in exudates and will be given further study. However, once the activatable system has been depleted, new increments of free streptokinase alone produce very little new active fibrin-lysing elements.

The development of specific antifibrinolytic antibodies following acute streptococcal infections has been previously demonstrated (15, 16). That the injection intrapleurally of streptococcal concentrates containing streptokinase also evokes the specific antistreptokinase response has been mentioned by Christensen (1), and was observed in connection with the earlier injections which were made primarily to estimate degrees of toxicity.

In Figure 3 evidence is presented from five cases of the development of antistreptokinase antibodies that were demonstrable in pleural exudates and followed intrapleural injections of streptococcal concentrates.

The rise in titre in the exudates occurred between the sixth and 11th days and was noted simultaneously in the serum.

The exact significance of the presence of specific antibody in nullifying the action of the fibrin-lysing system in local areas is not at present clearly established. Subsequent reference will be made to this point in connection with patient A. M. of Figures 7 and 8.

Figure 4 is presented for the purpose of demonstrating the total findings with respect to their correlation obtained serially in a single patient. In addition to the data described in Figure 1 (fibrinogen and N.P.N.) and Figure 2 (activatable fibrin-lysing system, active fibrin-lysing system, and free streptokinase) and Figure 3 (specific antistreptokinase), measurements of total protein and formol titratable substances are included.

The patient, J. G., had acute fibrinous pleurisy of tuberculous origin which had been active for ten weeks when first studied. He received two injections of streptococcal concentrate, the first con-

taining 100,000 units and the second, three days later, containing 200,000 units of streptokinase.

The individual findings are comparable to those presented in Figures 1 to 3 and they demonstrate the rising and falling changes previously described. The fibrinogen drops and the N.P.N. rises; the activatable fibrin-lysing system drops as it is rapidly transformed into the active fibrin-lysing system which attacks the fibrinogen and the local deposit of fibrin; antistreptokinase being evident in increased titre in the exudate on the 11th day.

The *total proteins* have exhibited no quantitative changes. They have been determined in grams rather than milligrams as has been the case with fibrinogen. This is in agreement with Christensen's findings (unpublished) of little or no *in vitro* digestion of serum albumins or globulins.

The formol titrations which have risen coincident with the rise in N.P.N. after each injection were of the same magnitude and probably represent further evidence of the intrapleural breakdown of fibrinogen and fibrin.

The findings in the case of patient J. G. of Figure 4 are similar to those obtained in each of the other cases of Group I. In addition to the results noted in Figures 1 to 4, the following observations have been noted in this group of patients. The pH of the chest fluids fell significantly (0.2 to 0.3 units) in 24 hours after the injection of the streptococcal concentrate, slowly returning to the control values, and has been considered as part of the phenomenon of increased proteolysis. Small but significant falls in viscosity were noted, which are consistent with the breakdown of the long eccentric fibrinogen molecules and depolymerization of any small amounts of desoxyribosenucleic acid that may be present. Twenty-four to 48 hours after injection, the sediment increased from 0.5% to 2.0%, then slowly decreased over the next two to ten days to the control levels. This appeared to be directly related to the increased cellular content occurring in the pleural fluids in association with the local irritation. As noted earlier, no significant changes in the amount of trypsin inhibitor were found in the samples of pleural fluid. Finally, the values for activatable fibrin-lysing system, trypsin inhibitor and antistreptokinase were found to agree

TH. - EMPYEMA

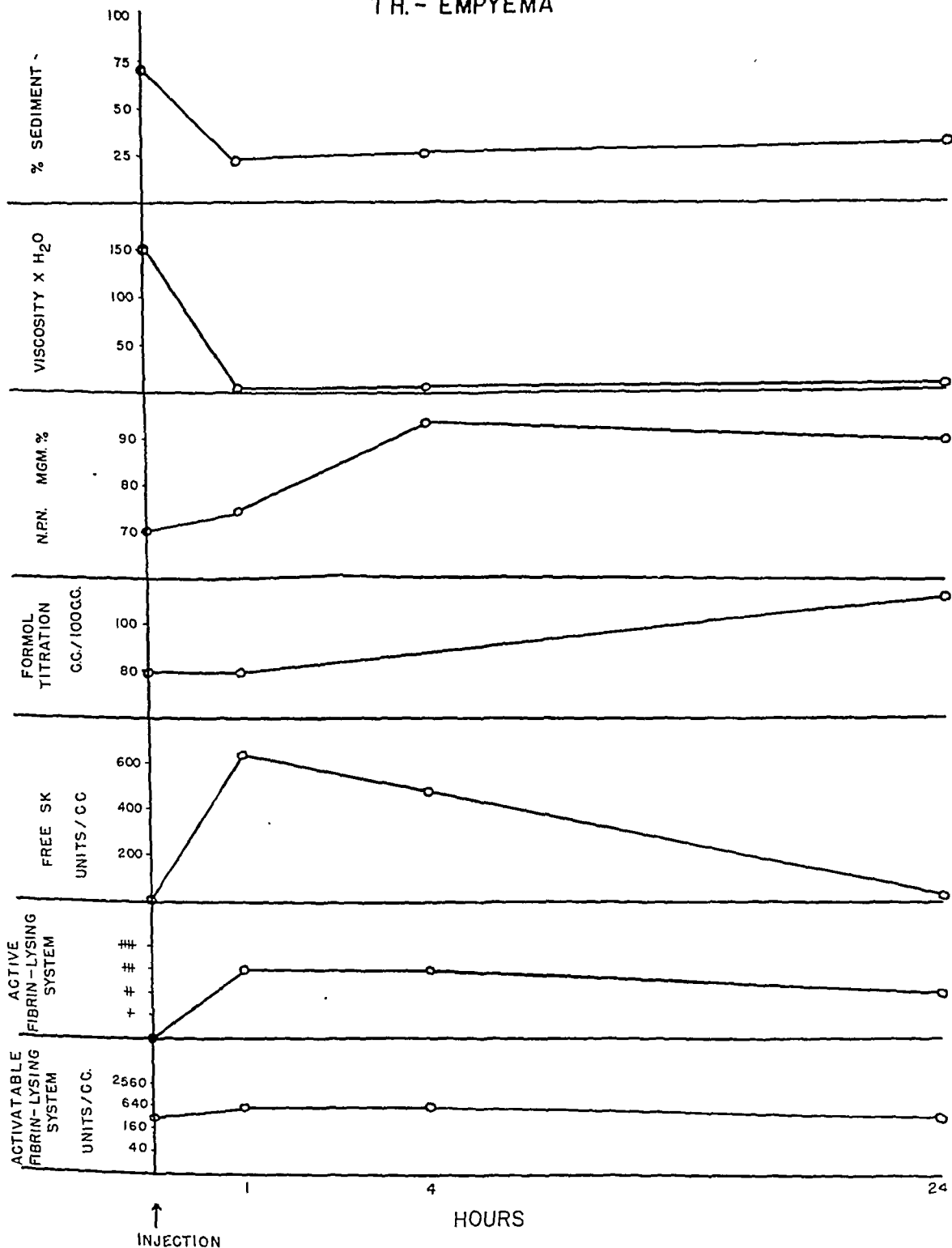


FIG. 5. THE EFFECT OF STREPTOCOCCAL CONCENTRATE (CONTAINING APPROXIMATELY 160,000 UNITS OF DESOXY-RIBONUCLEASE AND 400,000 UNITS OF STREPTOKINASE INJECTED INTRAPLEURALLY), IN A CASE OF MIXED TUBERCULOUS EMPYEMA ON THE ACTIVATABLE AND ACTIVE FIBRIN-LYSING SYSTEMS, FREE STREPTOKINASE, FORMOL TITRATION, N.P.N., VISCOSITY, AND SEDIMENT OF THE CHEST FLUID

The striking change in the character of the fluid is seen in the accompanying photo (Figure 6).

closely for both chest fluid and serum prior to the injection of streptococcal concentrates. The decrease noted in the activatable fibrin-lysing system after streptokinase injections occurred only locally since no changes in this value occurred in the serum at any time. Antibody changes, however, as previously described, were evident simultaneously both locally and in the general circulation.

Group II. Cases of bacterial empyema, Eight patients.

Four cases of mixed infections associated with tuberculous empyema and bronchopleural fistula.

One case of empyema due to Friedlander's bacillus.

One case of pneumococcal (Type XVII) empyema.

One case of post-pneumonic empyema (sterile).

One case of empyema due to anaerobic streptococcus.

Interesting differences were observed between the constituents of purulent empyemal fluid and those of acute fibrinous pleurisy. For example, fibrinogen has not been found to be present in the purulent material aspirated before the injection of the concentrates. The supernatant portion of centrifuged specimens failed to produce coagulum when thrombin was added. It seems probable that the formation of fibrin had previously occurred to a considerable degree at the site of infection and depleted the exudate of its fibrinogen.

In the empyemal group, the presence of desoxyribose nucleoprotein as a significant portion of the granular sediment was regularly noted, and, as previously reported (12), constituted from 30% to 70% of the total solids. In addition, although not recorded in detail in this article, preparations stained by the Feulgen method have revealed the presence of nucleoprotein occurring extracellularly as fibrous reticulum, granules, and amorphous plaques.

In Figure 5 comprehensive data are presented concerning one of the patients with empyema. The patient, T. H., had chronic pulmonary tuberculosis, with a left bronchopleural fistula, draining thoracotomy, and a mixed empyema. After aspirating a sample of pus, streptococcal concentrate containing approximately 160,000

units of desoxyribonuclease and 400,000 units of streptokinase in 10 cc. of saline were injected. The rapid and extensive liquefaction of the nucleoprotein by the desoxyribonuclease was demonstrated by the marked fall in viscosity from 150 \times water to 3 \times water and decrease in measurable sediment from 70% to 23% in one hour. These quantitative changes persisted over the 24 hours of sampling. Subsequent drainage of the empyema was temporarily facilitated following the liquefying changes. However, the lysing procedure was not continued and consequently the clinical value was not extensively tested.



FIG. 6. RAPID LYSIS BY STREPTOCOCCAL CONCENTRATE OF PURULENT PLEURAL EXUDATE WITHIN PLEURAL CAVITY OF PATIENT

Patient T. H. Chr. Pulm. Tbc, lt. bronchopleural fistula, pyopneumothorax and thoracotomy. Streptococcal concentrate containing desoxyribonuclease and streptokinase injected intrapleurally 12/4/47.

Specimen at left: Taken immediately prior to injection. Ten cc. centrifuged for one hour. Sediment 70%—thick, pink, viscid, and containing large cheesy shreds. Viscosity of whole fluid 150 \times water.

Specimen at right: Removed from chest one hour after injection. Ten cc. centrifuged for one hour. Sediment 23%—separates readily into three layers. From bottom up: small viscid layer; large pink layer of mixed red and white blood cells; and white layer composed of white cells and granules. Viscosity of whole fluid 4 \times water.

Definite increases in N.P.N. and formol titratable material were also observed. One hour following the injection the free streptokinase titre was 640 units/cc., and subsequently fell to 10 units/cc. in 24 hours. As in the cases previously described, the active fibrin-lysing system appeared but to a less degree than in the fibrinous pleuritic group.

Even though the above data indicate some degree of activity of the fibrinolytic system, the findings in this patient demonstrate the significant action of the streptococcal desoxyribonuclease. The changes in the physical character of the empyemal pus are strikingly illustrated in the photographs shown in Figure 6. The amount of lysis of sediment that occurred at the site of infection within the patient's thorax is evident and it was to the greatest degree due to the depolymerization of the nucleoprotein of the pus by the nuclease contained in the concentrate that was introduced.

Similar findings were obtained in six of the eight cases of empyema. One of them is worthy of further mention. The patient, T. P., entered the hospital with acute lobar pneumonia of four days' duration. He was treated with penicillin but his course and X-ray were suggestive of empyema. Aspiration of the chest performed on the tenth day of hospitalization yielded only a few cubic centimeters of thick green fluid which was sterile on culture. Introduction of air intrapleurally revealed the presence of several loculations within the empyemal cavity. Ten days later, 50 cc. of thick greenish pleural fluid was withdrawn and streptococcal concentrate containing approximately 40,000 units of desoxyribose nuclease and 100,000 units of streptokinase in 10 cc. of saline was introduced into the site of aspiration. Examination of specimens of fluid obtained by subsequent repeated aspirations revealed that the viscosity of the pus fell from $38 \times$ water to $14 \times$ water in one hour, and to $5 \times$ water in 24 hours. The sediment decreased from 35% to 14% in one hour, and to 10% in 24 hours. The N.P.N. rose from 58 mgm.% to 73 mgm.% in one hour, and to 84 mgm.% in 24 hours. Twenty-four hours after the injection 465 cc. of thin greenish-grey fluid was removed with ease. An X-ray of the chest at this time revealed that the latest aspiration had effected almost complete drainage of

the empyemal cavity and that the previous evidence of loculation had disappeared. Following this tap, the temperature fell rapidly to normal, the leucocytosis disappeared within a few days, and the patient made an excellent clinical recovery.

In one case of mixed empyema and in the one due to Friedlander's bacillus infection, the pHs of the fluids were found to be very acidic (5.50, and 5.21) apparently due to extensive proteolysis and digestion (N.P.N.'s 184, and 400) that had occurred spontaneously as a result of the diseases themselves. The injected streptococcal concentrate had no effect due to the low pH since studies, *in vitro*, have shown that both streptokinase and desoxyribonuclease are rapidly inactivated at this pH.

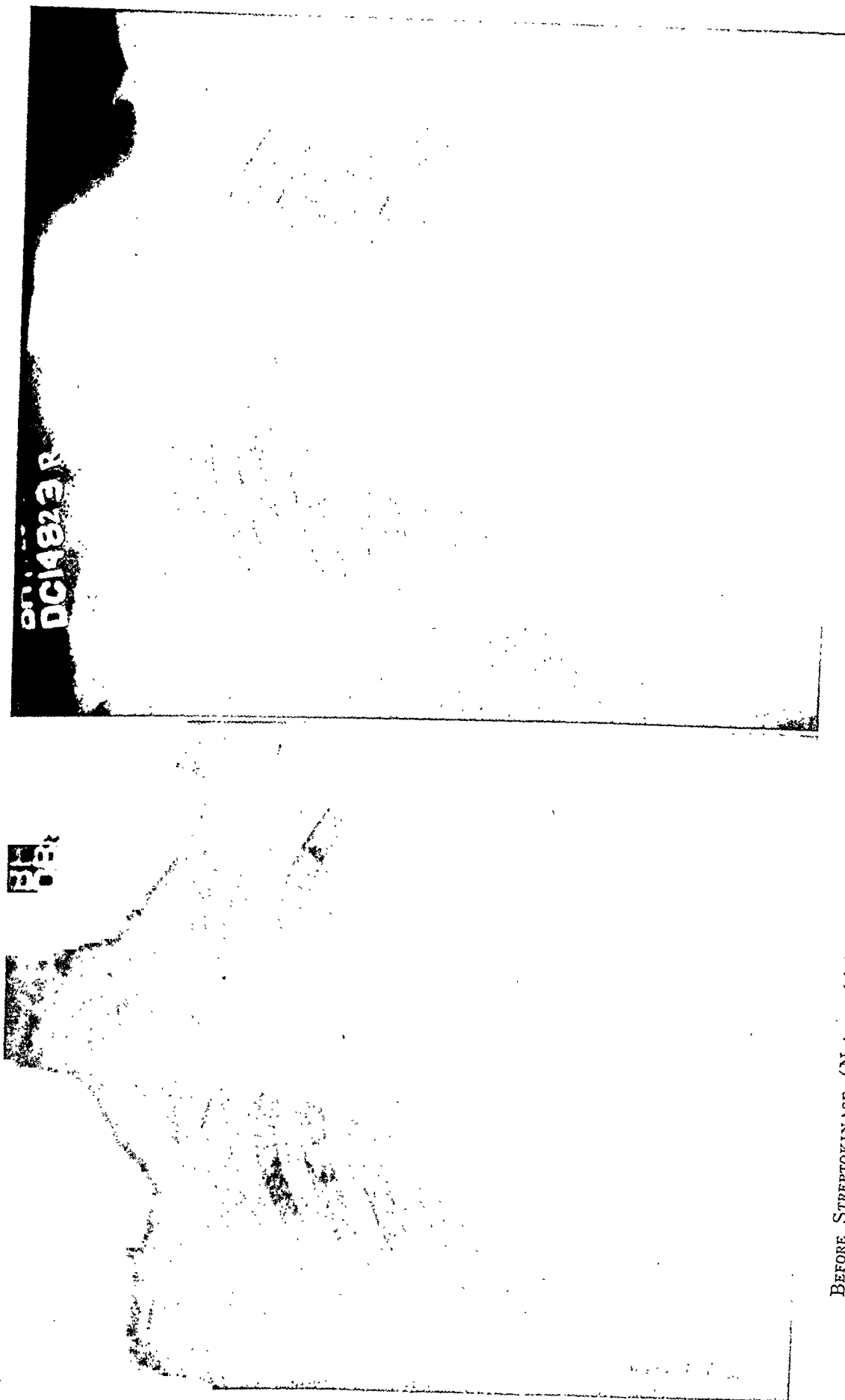
In cytological studies of the sediment of exudates, both of the acute fibrinous and the purulent groups, one feature of special interest has been the consistent breaking up of clumps of white blood cells. This change has been evident in the contrast between the cells of the pre-injection specimen and those of the sample obtained one hour after the introduction of the streptococcal concentrate. Whether or not the clumping was due to the fact that the cells were made adherent by a coating of fibrin or fibrous-nucleoprotein is not yet established. However, since the concentrates contained both streptokinase and nuclease a final explanation of the cellular effect awaits further study. As mentioned previously a detailed report of the cytological studies will be the subject of an early communication.

From the results obtained in the observations on the empyemal cases there is ample evidence that nucleoprotein depolymerization and probably fibrinolysis occurred, the former by the extensive decrease in the nucleoprotein sediment and fall in viscosity, the latter by changes in the fibrin-lysing system and changes in the N.P.N. and formol titration.

Group III. Cases of hemothorax, two patients. In each, the intrathoracic blood coagulum developed following pneumonectomy.

Figures 7 and 8 illustrate changes in a case of loculated hemothorax that were brought about by the streptococcal fibrin-lysing system.

400,000 units of streptokinase were introduced into a single pocket containing 71 cc. of



BEFORE STREPTOKINASE (Note multiple fluid levels)

AFTER STREPTOKINASE (Note all loculations due to fibrinous bands have been eradicated)

Patient A. M. 20 yrs. old. Left pneumonectomy for cystic bronchiectasis. Postoperative course complicated by persistent fever and loculated hemothorax preventing aspiration of contents and subsequent thoracoplasty. 400,000 units streptokinase were introduced intrapleurally into small pocket. In 24 hours, chemical methods (chest fluid volume determinations) indicated conversion of small pocket into a single large cavity. In next two days 1,300 cc. of sanguinous fluid were removed readily from one site, and X-ray revealed that pockets were gone. Thoracoplasty followed shortly with good results.

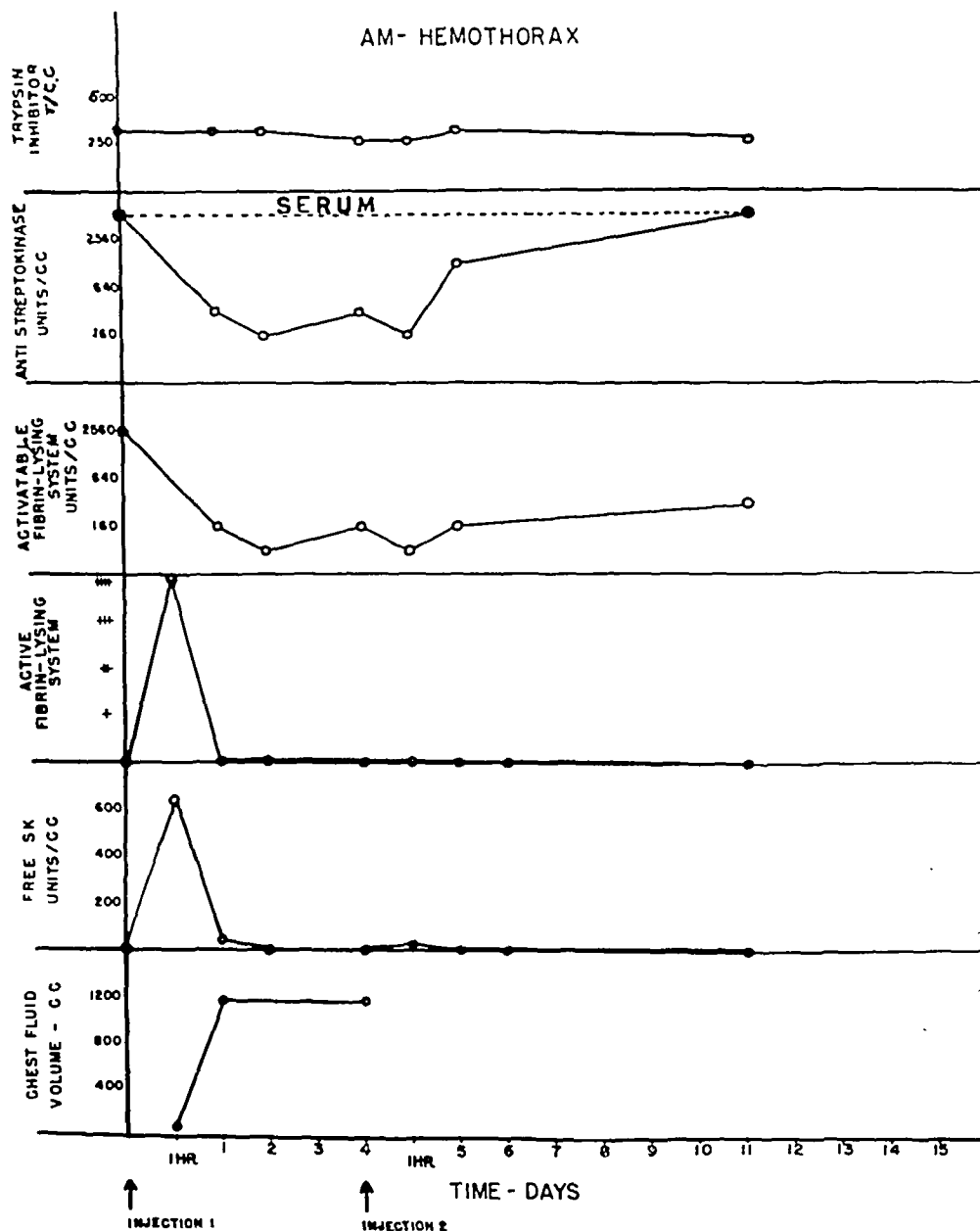


FIG. 8. THE EFFECT OF TWO INTRAPLEURAL INJECTIONS OF STREPTOCOCCAL CONCENTRATE IN A CASE OF POST-OPERATIVE HEMOTHORAX ON THE CHEST FLUID VOLUME, FREE STREPTOKINASE, ACTIVE AND ACTIVATABLE FIBRIN-LYSING SYSTEMS, ANTISTREPTOKINASE, AND TRYPSIN INHIBITOR OF THE CHEST FLUID

400,000 units of streptokinase were given in injection 1, and 200,000 units in injection 2.

The striking clinical change is described in the text, and seen in the accompanying X-rays (Figure 7).

fluid. The concentration in this isolated area was, therefore, 5,600 units/cc. before resolution of the loculating bands took place.

The legend under Figure 7 gives the essential details of the findings in this patient both before and after the introduction of streptococcal concentrate and illustrates objectively the fibrinolytic

effect. In this instance the postoperative coagulum prevented drainage of the intrathoracic contents. However, after dissolution of the fibrinous bands and a considerable amount of the associated blood clot it was possible during the ensuing 48 hours to drain by thoracentesis a large proportion of the hemothorax which had become liquefied.

The multilocular condition had been transformed into a unilocular state. It seems likely that the fibrinolytic system was chiefly operative.

It is of special interest to note that this patient had a high titre of antifibrinolysin (antistreptokinase) at the beginning of the study. Although not proved by cultures it seems likely that he had previously had one or more infections with hemolytic streptococci since the height of antibody titre is consonant with that previously found in patients convalescent from known infection with hemolytic streptococci.

In spite of the presence of immune bodies, however, impairment of the action of the streptococcal product was not apparent. It is not possible at the present time to offer an explanation of these findings. However, the possibilities suggest themselves either that a sufficient amount of streptokinase was injected to bind with antibody and leave an excess, or that the active lysing system was put in operation before the antibody took effect. Further study of this phase of the problem is required. Also evident from the data, as in the results with the previous cases, is the fall in the activatable fibrin-lysing system coincident with the appearance of an active fibrin-lysing system, and its slow return toward the control value. Trypsin inhibitor did not change despite the activation and rapid inactivation of the fibrin-lysing system.

The course in the patient just described appears to represent an example of a beneficial therapeutic result effected by the action of the streptococcal fibrin-lysing system.

The second patient with postoperative hemothorax will be referred to only briefly since he is still under observation. Thirty-two days postoperatively the patient was transferred to our Service because it was impossible to obtain more than a few cubic centimeters of fluid from the postoperative side of the thorax. X-ray examination revealed a large shadow with fluid levels occupying approximately one-half of the thoracic cavity. From three aspirations at different sites a total of 3 cc. was obtained. Into each of the sites streptokinase was injected up to a total of 250,000 units. In the preparation of concentrate employed in this case almost all of the nuclease had been eliminated. Consequently only the fibrinolytic system was operative to any significant

degree. Twenty-four hours later 600 cc. of thin brownish fluid were removed by thoracentesis from a single site without difficulty. Immediately after the aspiration, by fluoroscopy and X-ray, only a small amount of effusion remained. Although fluid reaccumulated over the next few days, successful aspirations were readily performed indicating a considerable reduction in the presence of loculation, and the creation of a favorable situation for further intercostal drainage.

DISCUSSION

A group of unique enzymatic activities have been utilized to produce changes in fibrinous, purulent and sanguinous exudations within the pleural cavities of patients. Thus through the mediation of substances elaborated by hemolytic streptococci, fibrin is caused to undergo lysis; fibrinogen is altered so that it no longer can assume the solid form of fibrin; and the coarse sediment of purulent exudate (primarily desoxyribose nucleoprotein) is degraded to a thin solution.

Once it had been well established by detailed preliminary observations that the methods of purification of the streptococcal products developed extensively by Christensen (1) yielded preparations that were progressively less toxic, although maintaining constant or increased potency, a demonstration of the occurrence and degree of action in patients of the active principles—streptokinase and desoxyribose nuclease—became a center of interest. For this purpose quantitative estimations were made of the results of the action of each enzymatic system on its respective substrate in the areas of disease. The rapidity of the beginning of action, and the duration of continuing action after a single, and in some instances after a second injection, were determined.

Definitive enzymatic changes of a significant degree were regularly obtained and were demonstrable within an hour after the injection and endured for several days before the effect subsided and disappeared. The phenomenon was a self-limiting one following each injection.

The clinical courses of the patients have been followed by frequent physical, laboratory, and X-ray examinations over extended periods. Following the injections a febrile response occurred

not infrequently and there was also evidence of local irritation. Both signs of reaction were, however, transient.

Since the periods, in which the action of the concentrates was operative, were limited to a few days, striking evidence of favorable alterations of a permanent nature was not expected nor definitely demonstrated except for the results obtained in cases of loculated hemothorax, one example of which is described in detail, and in some of the instances of empyema. In patients with hemothorax, it is obvious that consideration must be given to the broad principles and problems of thoracic surgery before the application of the procedure may be most advantageously made.

The findings, however, constitute a background against which the scope of the study is being broadened to include a consideration of the possible usefulness of the procedure when utilized under appropriate circumstances. The desirability of causing the liquefaction of fibrin or of preventing its formation, or of resolving purulent nucleoprotein containing sediments, would on theoretical grounds depend upon a variety of conditions associated with the pathogenesis and expected evolution of the disease processes to which this method involving lytic activities might be applied. Comparable principles would also be applicable to exudative diseases involving locations in the body other than the pleural area.

If the solid increments constitute part of an advantageous walling off process then the liquefaction of the wall might promote a spread of the infection in its acute phases before the patients' immunity was sufficient to restrain the dissemination of the infection. On the other hand, the same walling off may prevent the introduction of antibacterial reagents of an immunological nature, as well as antibiotic substances, into the field of disease. Under the latter circumstances elimination of the wall might be advantageous. Other sets of conditions concern factors that are involved in the ultimate formation of scar tissue, or of adhesions, and the permanent thickenings that are related to the final organization of infected areas.

The results presented in this article serve as a basis for extending the study along the lines that have been discussed.

SUMMARY

1. Twenty-three patients suffering from exudative pleuritis associated with different types of diseases have received intrapleural injections of partially purified concentrates containing streptococcal fibrinolysin (streptokinase) and desoxyribose nuclease.

2. Intrapleural fibrinolytic and proteolytic changes due to the activity of the fibrin-lysing system were demonstrable in samples of the exudates taken at repeated intervals after the injection. The effects were self-limiting after each injection.

Intrapleural depolymerization of the nucleoprotein of the solid sediment of the exudates was also demonstrable in a similar manner.

3. When toxic manifestations occurred following the injections, they were limited to transient febrile reactions with general malaise, and a local outpouring of leucocytes of a few days' duration.

4. The possibility is discussed of influencing favorably by the enzymatic systems the course of exudative types of diseases and it is being given additional study.

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THE BEHAVIOR OF RADIOIODINE IN THE BLOOD¹

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Radioiodine has been used to study the function of the human thyroid gland in various ways. The quantity of radioiodine collected by the thyroid has been determined directly by *in vivo* measurements (Hamilton, Soley and co-workers [1-4]; Hertz and co-workers [5]), and indirectly by estimations from the total quantity excreted in the urine over a fixed period (Hertz and co-workers [5]; Rawson and co-workers [6, 7]). The distribution of iodine in the thyroid has been studied by autoradiograms (3) or by chemical analysis of excised tissue (5). Most recently the capacity of the thyroid to concentrate radioiodide has been studied by Stanley and Astwood (8) after previous administration of an antithyroid drug such as mercaptimidazol.

Keating, Power, Berkson, and Haines (9) have employed radioiodine to investigate the kinetics of iodine metabolism. They studied the urinary excretion of radioiodine by collecting all the urine in short periods for three days or longer. By plotting cumulative urinary excretion against time, they obtained an exponential curve from which were estimated four quantities: (1) a renal fraction (that part of the dose of radioiodine primarily excreted in the urine); (2) a disappearance rate (which they inferred to represent the proportional rate of disappearance of radioiodine from the blood); (3) a renal excretion rate (the proportional rate of excretion into the urine); and (4) a so-called collection rate (the proportional rate of disappearance into other sites than the kidneys, of which the most important is the thyroid). The most significant measure of thyroid activity was the collection rate, which was much less than normal in hypothyroid

patients and much greater than normal in hyperthyroid patients.

The present study was undertaken as an extension of the foregoing observations. Its objects were (1) to examine directly the behavior of radioiodine in blood after its oral administration to persons who had varying states of thyroid function; (2) to compare the disappearance rate of radioiodine in blood as determined directly from observations in the blood itself with estimates of this same value obtained from urinary studies in the manner previously described; and (3) to examine the appearance in the blood of organically bound radioiodine.

PROCEDURES

The subjects selected for study came to the laboratory without breakfast. After withdrawal of a control sample of blood, radioiodine (I^{131} , Oak Ridge Laboratories, A. E. C.), in the form of sodium iodide dissolved in about 150 cc. of water containing 100 μ g. of nonradioactive sodium iodide, was given by mouth. Samples of blood and urine were thereafter collected at stated intervals. The quantity of radioiodine present in these specimens was estimated by means of a Geiger-Müller counting tube with thin mica window in connection with a suitable scaling circuit. All counts were corrected for "background," which was uniformly about 0.5 count per second or less. Appropriate aliquots of each dose given were reserved as standards, each of which was counted simultaneously with its corresponding blood and urine samples. The method of preparing urine for counting has been described (Keating and co-workers [9]). The blood samples were allowed to clot at room temperature and, after the separation of the serum by centrifugation, were analyzed for radioactivity as described in the next section.

Total radioactivity of serum. The counting of films of serum dried directly on thin copper disks was unsatisfactory. However, after partial alkaline hydrolysis dry films suitable for counting could be obtained. One cc. of serum in a pyrex test tube 7 mm. (internal diameter) by 100 mm. was thoroughly mixed with 0.100 cc. of 2.5 N solution of sodium hydroxide. The tube was covered with a small pyrex cup and heated for 30 minutes at 10 to 15 pounds in a pressure cooker. This treatment caused little or no change in volume. Some sediment which

¹ An abridgment of a portion of the thesis submitted by Dr. McConahey to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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formed was carefully resuspended by shaking, although later studies showed that little or no error was introduced by failure to resuspend this sediment. Samples of the alkaline digest were measured out for radioactivity analysis, by means of a syringe pipet calibrated to deliver 0.200 cc. In order to prevent accumulation of radioiodine in this pipet, which might then cause error in future samples, it was rinsed between measurements with 10 portions of strong solution of potassium iodide followed by 10 portions of water. Finally two portions of the digest to be examined were drawn in and discarded, then duplicate 0.200 cc. samples were transferred to copper disks 1 inch (2.5 cm.) in diameter. The samples were dried slowly under an infra-red lamp, cooled, and counted immediately thereafter, in order to avoid variations attendant on the possible absorption of moisture by the dry alkaline films. The counts observed, corrected for background as mentioned previously, were converted to counts per second per unit volume and, by reference to the simultaneously counted standard, to percentage of administered dose per liter of serum. Each planchet was counted at least twice, and usually enough counts were taken so that the variation due to randomness of atomic disintegration would be less than 5 per cent. Finally, a positive correction of 15 per cent was applied to all counts of serum digests, to compensate for losses due to "absorption" of beta rays by the deposit of alkali and partly degraded protein.

This correction factor was obtained as follows: To 34 samples of normal serum, each measuring 0.9 cc., were added 0.1 cc. of radioiodine of known radioactivity and 0.1 cc. of 10 per cent sodium hydroxide. The samples were heated in a pressure cooker and 0.2 cc. aliquots were prepared for dry counting on planchets as described previously. These were compared with planchets prepared from equal amounts of radioiodine in aqueous solution as standards. It was found that in the 34 samples, the mean loss of radioactivity by absorption was 15.2 ± 0.5 per cent with a range of 11.1 to 20.0 per cent.

In order to investigate the reproducibility of the method, blood was drawn from a hyperthyroid patient 24 hours after administration of 100 microcuries of I^{131} . Ten samples of the serum, each of 1.0 cc., were analyzed for their radioactivity. The mean value obtained was 11.19 counts per second per cubic centimeter. The standard deviation for the determinations from the separate specimens of serum was 0.43 counts per second or about 4 per cent of the mean, which gives an estimate of the error of such determinations. Taking into consideration variations caused by "absorption," variations inherent in the counting equipment and errors introduced by chemical manipulation, it is estimated that the over-all experimental error of a single determination of radioiodine did not exceed 10 to 15 per cent.

Radioiodine precipitated from serum by protein precipitants. This fraction, henceforth referred to as "precipitable" radioiodine, was separated by means of precipitation of proteins with the zinc sulfate-sodium hydroxide reagents of Somogyi (10, 11), as used by Man, Smirnow, Gildea and Peters (12) in their study

of serum iodine fractions. In this procedure, 1 cc. of serum in a 15 cc. pyrex centrifuge tube calibrated in tenths was treated with 1.00 cc. of 0.75 N solution of sodium hydroxide and 8.00 cc. of 0.125 per cent zinc sulfate in 0.031 N solution of sulfuric acid. The mixture was stirred thoroughly with a thin glass rod and centrifuged for five minutes at 1,500 r.p.m., and the supernatant was removed by decantation. The tube was filled with distilled water, the protein precipitate was thoroughly dispersed by stirring with the glass rod and the tube was again centrifuged. The supernatant was again discarded and the washing procedure was repeated twice more. The precipitate was next dispersed by stirring with 0.50 cc. of 2.5 N sodium hydroxide. The stirring rod was rinsed with two drops of water, and the alkaline mixture was autoclaved as in the determination of total radioiodine. After removal of the tube from the cooker, the volume of the digest was noted, and 0.200 cc. samples were measured out, dried and counted, as previously described. "Absorption" losses in this method of determining serum precipitable radioiodine were about the same as those noted in the determination of total radioactivity (actually the mean loss of 15 samples was 15.7 ± 0.6 per cent). Control experiments in which radioiodine was added to serum showed that all but 0.25 to 2.9 per cent of added radioactivity was removed by the precipitation and washing technic.

An estimate of the error of the method for serum precipitable radioiodine was obtained in the following manner: ten 1.0 cc. samples of serum from blood which had been withdrawn from a hyperthyroid patient 24 hours after oral ingestion of a tracer dose were analyzed for serum precipitable radioiodine. The mean value obtained was 2.13 counts per second per cubic centimeter and the standard deviation for the determination from the separate serum specimens was 0.36 counts per second or about 17 per cent of the mean.

Miller and associates (13) found that exchange occurred *in vitro* between inorganic and organic iodine compounds under certain conditions of pH and temperature. The possibility that similar exchange might account for some of the precipitable radioiodine observed with the method which we employed was investigated in the following manner. Radioiodine as iodide was added to each of eight tubes containing 1.0 cc. of normal human serum. Four tubes were stored at room temperature, and four were incubated at body temperature. One specimen from each group was analyzed for serum precipitable radioiodine after four, six, eight and 13 days, respectively. The quantity of radioiodine remaining in the precipitable fraction after washing was negligible (0.2 to 1.5 per cent of the total radioiodine present in the whole serum). On the basis of these observations it was felt that exchange could not account for any significant proportion of the precipitable radioiodine observed.

Thirty-one persons who had various thyroid conditions consented to be studied; 28 of these were patients being treated for thyroid disease and three were normal subjects. Serial blood levels of total radioiodine were determined in all 31 persons. Table I shows the functional

TABLE I

Types of persons studied and the variations of the basal metabolic rate

States of thyroid	Cases	Basal metabolic rate
		per cent
<i>Hyperthyroidism</i>	17	
Exophthalmic goiter	16	
Primary	5	+40 to +53
Recurrent	11	+15 to +60
Adenomatous goiter with hyperthyroidism	1	+21
<i>Euthyroidism</i>	9	
Normal subjects	3	Not done
Adenomatous goiter without hyperthyroidism	4	-14 to +5
Carcinoma of thyroid	2*	-4
<i>Hypothyroidism (myxedema)</i>	6*	-36 to -11

* One patient who had carcinoma of the thyroid was studied twice—once while he was in a euthyroid state and once while he was myxedematous as a result of a "thyroidectomizing dose" of I^{131} .

state of the thyroid and basal metabolic rates of the patients studied. Seventeen had hyperthyroidism, nine were euthyroid and six had myxedema. One patient who had carcinoma of the thyroid was studied twice—once while his thyroid function was normal and later while myxedema was present as a result of a "thyroidectomizing dose" of I^{131} . By a euthyroid person is meant one whose tissues at large receive the normal amount of thyroid hormone daily irrespective of the anatomic state of the thyroid gland.

Of the 36 doses of I^{131} given, 21 were tracer and 15 were therapeutic doses. The tracer dose used in most cases was 100 microcuries, although a few patients received more. A therapeutic dose is here defined as one large enough to destroy thyroid tissue by radiation. Comparison of tracer doses with subsequent therapeutic doses in the same patient did not disclose consistent or significant differences which could be ascribed to radiation effect within the 48-hour period when most observations were made. This does not exclude the possibility that radiation effects may have been present in either the tracer or the therapeutic doses, but it appears reasonable to assume that any effects of radiation present in the data are of small magnitude.

The use of 100 μ g. of sodium iodide as carrier in these studies theoretically introduces an error in the tracer character of the experiments. It may be calculated that in the average person this quantity of iodide in equilibrium with the blood and extracellular fluids represents an initial increase in the concentration of iodide of the order of 0.5 μ g. per 100 cc. of plasma. We have compared studies conducted with 100 μ g. of carrier with studies employing carrier-free radioiodide as well as

studies involving a carrier of 1 to 10 μ g. Such observations indicate that the use of 100 μ g. of sodium iodide as carrier does not affect the results observed either qualitatively or quantitatively to any significant degree.

RESULTS

Total radioiodine in blood serum. Radioiodine appeared in the circulating blood within a few minutes after oral administration. The concentration of radioiodine rose rapidly and reached a maximal level within one-half to one and one-half hours after ingestion, the time at which the

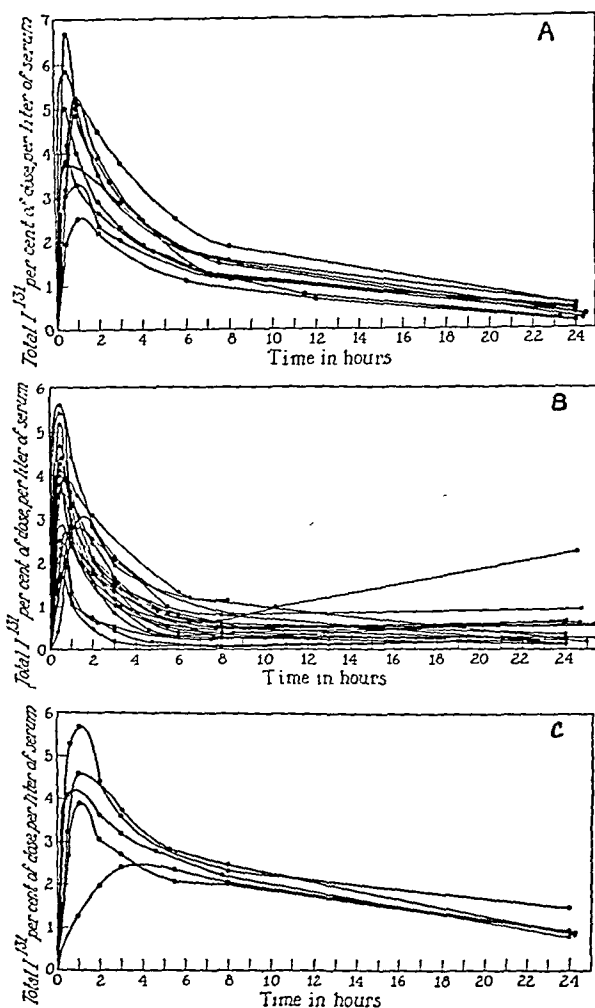


FIG. 1. THE CONCENTRATION OF TOTAL RADIOIODINE IN THE SERUM OF PERSONS WHO HAD (A) NORMAL THYROID FUNCTION, (B) HYPERTHYROIDISM AND (C) MYXEDEMA

The group who had normal thyroid function includes three normal persons, four patients who had adenomatous goiter without hyperthyroidism, and two patients who had low grade nonfunctioning carcinomas of the thyroid.

maximum level was attained varying somewhat with the functional state of the thyroid. Thereafter, the concentration diminished at a rate which was related to the functional state of the thyroid. Figure 1 shows the individual curves for the concentration of radioiodine in serum during the first 24 hours in all cases of the three groups studied. Figure 2 shows average curves for each of the three groups. In euthyroid patients, the concentration of radioiodine in the blood reached a maximal level between one-half and one hour and then fell for the rest of the 24 hours. In hyperthyroid patients, the concentration reached a maximal level sooner, usually in about one-half hour, then fell more rapidly than in the normals for six or eight hours, and thereafter decreased very slowly in some cases, leveled off in others, and in a considerable number again rose a second time from the low value observed at about eight hours after the dose. In patients who had hypothyroidism, a maximal level was reached later, usually between one, and one and one-half hours, and the level thereafter diminished more gradually than in euthyroid persons. Since, as is well known, both hyperthyroidism and myxedema affect the volume of both plasma and extracellular fluid, it is apparent that these data must be modified to some extent by such factors. Accurate evaluation of these factors would be possible only if both blood volume and volume of extracellular fluid had been measured in each individual case, a procedure which we were unable to follow in this study. Fortunately it appears probable that

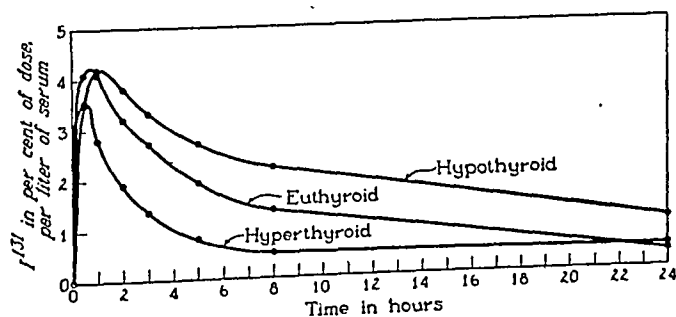


FIG. 2. COMPOSITE CURVES OF THE CONCENTRATION OF TOTAL RADIOIODINE IN THE SERUM IN VARIOUS THYROID STATES

Constructed from the mean concentrations for each group versus time. (From McConahey, W. M., Keating, F. R., Jr., and Power, M. H., The behavior of radioiodine in the blood in various thyroid states. *Tr. Am. A. Study Goiter* [In press].)

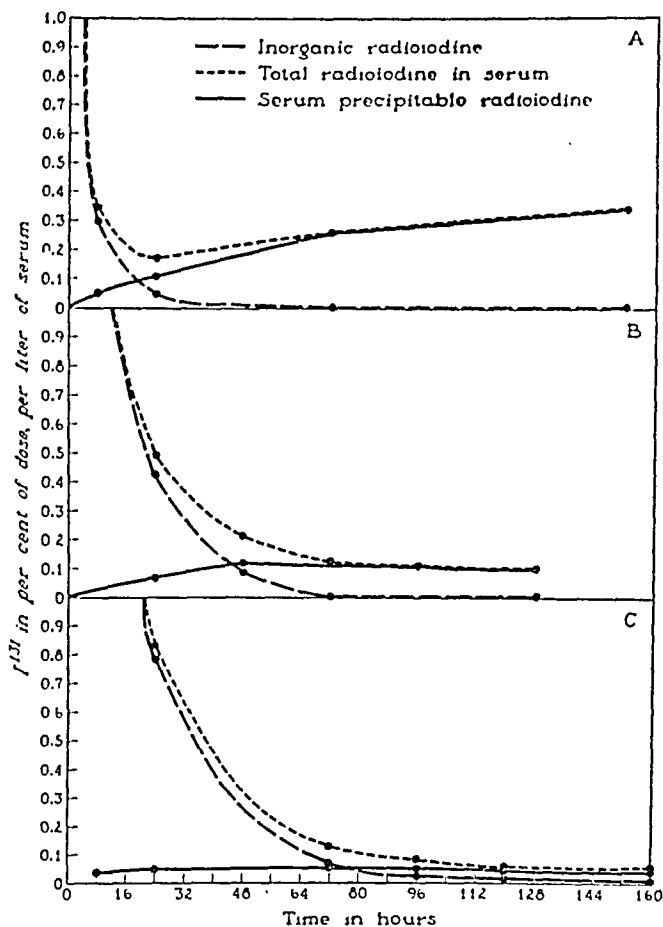


FIG. 3. SEPARATION OF THE CONCENTRATION OF TOTAL RADIOIODINE IN SERUM INTO A SERUM PRECIPITABLE FRACTION AND AN INORGANIC FRACTION

The latter was obtained by subtracting precipitable radioiodine from total radioiodine. (A) A case of hyperthyroidism. (B) A person who had normal thyroid function. (C) A case of myxedema.

changes in blood volume and the volume of extracellular fluid accompanying various levels of thyroid activity were relatively minor factors in the differences which we observed.

Serum precipitable radioiodine. Levels of precipitable radioiodine in serum were determined in 13 cases (nine hyperthyroid, one myxedematous, and three euthyroid patients). Since it was not possible with the technics employed to measure precipitable radioiodine in the blood with accuracy after the administration of a small tracer dose, only persons who had received larger doses of radioiodine were studied in this manner. The doses of I^{131} administered ranged from 1 to 100 millicuries. The results of this portion of the study may, therefore, have been affected by the consequences of radiation, but one may be justified in assuming that the results observed corre-

spond at least qualitatively to physiologic conditions.

Figure 3A shows the values for serum precipitable radioiodine plotted as percentage of dose per liter of serum for a case of hyperthyroidism. The curve of total radioiodine in the serum is broken into two fractions: the one of serum precipitable radioiodine and the other of inorganic radioiodide obtained by subtracting values for precipitable radioiodine from total radioiodine. In Figure 3B are plotted the corresponding data in a patient who had normal thyroid function. Serum precipitable iodine appeared more rapidly and in larger concentrations in the hyperthyroid patient than in euthyroid persons and this fact appears to account for the leveling off or for the secondary rise in the curve of total radioiodine in serum observed in hyperthyroid cases. Figure 3C shows total radioiodine concentration and the inorganic and serum precipitable fractions, in a myxedematous patient. Inorganic iodide falls more slowly and precipitable iodine reaches a smaller concentration than in normal persons. In hyperthyroid subjects apparently all of the circulating radioiodine was precipitable after a period which averaged about 48 hours (Figure 4). In euthyroid subjects the average time re-

quired for all circulating radioiodine to become precipitable was about 96 hours, and in the myxedematous subject this time was 290 hours. The foregoing results do not preclude the possibility that a small inorganic fraction might persist in any case for a longer time since the method employed for measuring precipitable radioiodine was relatively insensitive.

COMMENT

Figure 1 shows considerable variability of the curves of total radioiodine concentration in serum during the first six hours. Overlapping by members of the different groups is at a minimum at eight hours. In myxedema the concentration of radioiodine exceeded 2 per cent of the dose per liter of serum (from 2 to 2.5 per cent) at eight hours. The euthyroid and hyperthyroid curves overlapped a little at this time (1 to 1.9 per cent and 0.05 to 1.2 per cent, respectively), but the curves of neither group overlapped those of the cases of myxedema. Only one of the euthyroid curves was less than 1.2 per cent and only two of the hyperthyroid curves were more than 1 per cent.

Examination of the curve of concentration of total radioiodine in serum during the first 24 hours discloses at least three components: (1) a rapid rise to a maximal concentration and a brief, rapid fall; (2) a more prolonged fall at an exponential rate, and (3) a continued but much slower fall (or sometimes, in case of hyperthyroidism, a secondary slow rise to a higher level). Component 1 appears to reflect absorption from the gastro-intestinal tract and establishment of equilibrium between the blood and the body fluids; component 2 represents the disappearance of radioiodine as inorganic iodide from the blood and the body fluids into the urine, the thyroid and other sites of disposal; component 3 reflects in part the appearance in the blood of precipitable (that is, organically bound) radioiodine.

Keating and co-workers estimated the disappearance rate of radioiodine from the blood from the curve of excretion in the urine. The disappearance rate can be determined directly from the second component of the concentration curve of radioiodine in the blood. When the logarithm of

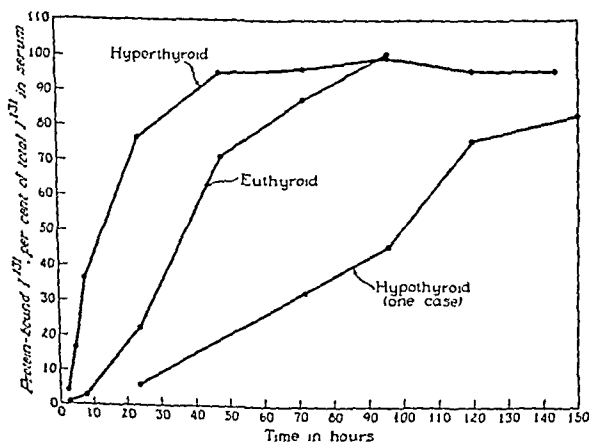


FIG. 4. SERUM PRECIPITABLE RADIOIODINE IN VARIOUS STATES OF THYROID FUNCTION

Each curve is constructed from the mean values of the cases comprising the group versus time; the value expressed is the percentage of the total radioiodine in serum which is precipitable. (From McConehey, W. M., Keating, F. R., Jr., and Power, M. H., The behavior of radioiodine in the blood in various thyroid states. *Tr. Am. A. Study of Goiter* [In press].)

concentration is plotted against time, the observations fit a straight line, the slope of which is the rate of disappearance of radioiodine from the blood.

The observations comprising component 3 fall above this straight line and in some hyperthyroid cases the period during which concentration of total radioiodine in blood fits an exponential function is rather short. If instead one plots the logarithm of inorganic radioiodine (estimated by difference as described) as ordinate, with time as abscissa, the points continue to fit an exponential function for a much longer period. This fact suggests that it is the inorganic iodide which disappears at an exponential rate and that it is the appearance of organically bound iodine which causes the curve of total radioiodine to deviate from an exponential curve.

TABLE II
Comparison of disappearance rate determined in blood and in urine

	Cases	Disappearance rate		Difference	P*
		Determined from blood	Determined from urine		
		<i>per cent/hr.</i>	<i>per cent/hr.</i>	<i>per cent/hr.</i>	
Euthyroid	9	$15.9 \pm 1.1^\dagger$	15.0 ± 0.8	-0.9 ± 1.3	
Hyperthyroid	17	40.2 ± 4.6	37.0 ± 4.7	-3.2 ± 2.7	0.2
Hypothyroid	6	8.8 ± 0.9	6.4 ± 0.6	-2.4 ± 0.8	0.04

* P values obtained from Table of T. R. A. Fisher, *Statistical Methods for Research Workers*, Seventh Edition, Oliver and Boyd, 1938.

† The values given are the means and standard errors of the means.

In Table II the mean disappearance rates of radioiodine from blood for each group, calculated directly from observations on the blood, are compared with the values obtained from simultaneous observations on the urine of each subject calculated in the manner described by Keating and co-workers. The means of the individual differences, which are also given, do not appear to be significant, and may reflect merely the errors of the methods employed. It seems, therefore, that the disappearance rate of radioiodine from blood measured by either method reflects variations in thyroidal function with respect to iodine collection. However, since radioiodine disappears from

blood into the urine, and also into other tissues in addition to the thyroid, disappearance rate is not a direct or quantitative measure of thyroidal accumulation of iodine. This is evidenced by the values for disappearance rate in cases of myxedema, in most of which accumulation of radioiodine by the thyroid was found by *in vivo* measurements to be negligible or absent.

The appearance of precipitable radioiodine in blood can be construed as largely representing radioiodine collected by the thyroid, synthesized into organic compounds and secreted into the circulation. Determinations of precipitable radioiodine reflect variations in thyroid function in several ways: 1. The concentrations of precipitable radioiodine reached in cases of hyperthyroidism are greater than in cases of euthyroidism. 2. Precipitable radioiodine can be detected sooner in hyperthyroid cases than in euthyroid cases and the concentration increases at a more rapid rate. 3. Estimation of precipitable radioiodine as a percentage of total serum radioiodine reflects both the foregoing and the differences in the behavior of inorganic iodide with variations in thyroidal function, so that precipitable radioiodine replaces the inorganic fraction much more rapidly than normal in hyperthyroid subjects and much less rapidly than normal in hypothyroid subjects. These relationships are entirely consistent with what is known about the behavior of chemically determined precipitable iodine in persons who have varying states of thyroidal activity.

It is surprising and a little disturbing to note the appearance of precipitable radioiodine in the single case of myxedema in which this determination was made (Figures 3C and 4). The patient was a young man having carcinoma of the thyroid who some months previously had been made myxedematous by means of a "thyroidectomizing" dose of radioiodine. The concentration of precipitable radioiodine observed in this case was small, and this concentration was substantially achieved within a few hours (Figure 3C). The survival of some thyroid tissue cannot be excluded, although no detectable collection occurred in the thyroid region and the basal metabolic rate was -25 per cent. The possibility of *in vivo* exchange occurring in the blood remains a remote possibility but such exchange could not

be demonstrated *in vitro*. The observations of precipitable radioiodine in this case may be of some interest in connection with the demonstration by Chapman (14) and by Morton and associates (15) that iodine may be organically combined elsewhere in the body than in the thyroid.

The method employed here to estimate concentration of precipitable radioiodine is relatively insensitive and relatively inaccurate. On this account and because of the possibility of distortion by the quantities of radiation involved it appears unwise to attempt to analyze quantitatively the kinetic behavior of organically bound radioiodine from these data. It appears likely, however, that with more sensitive and accurate methods of analysis the rate of increase of organically bound radioiodine in blood and the maximal concentration achieved can be compared with the concentration of protein-bound iodine measured by chemical methods in order to obtain a measure of turnover rate of protein-bound iodine.

SUMMARY AND CONCLUSIONS

1. Levels of total radioiodine in the blood after oral ingestion of I^{131} in the form of iodide show significant differences in various thyroid states. Eight hours after ingestion, mean concentration of radioiodine in the serum was 2.06 per cent of the dose per liter in cases of myxedema, 1.28 per cent in euthyroid persons and 0.49 per cent in cases of hyperthyroidism.

2. The curve of total radioiodine in serum plotted against time has three components: (a) an initial rapid increase followed by a brief, rapid fall, regarded as reflecting gastro-intestinal absorption and equilibration with body fluids; (b) an exponential fall, regarded as reflecting disappearance of radioiodine as iodide from the blood into the thyroid, the urine and other sites of disposal, and (c) a third phase which indicates the appearance in the blood of organically bound radioiodine.

3. The rate of disappearance of radioiodine from the blood reflects variations in thyroidal function, being more rapid than normal in hyperthyroid subjects and less rapid than normal in hypothyroid ones.

4. Comparison of the rate of disappearance of radioiodine from blood estimated directly from

blood itself with estimations of the same rate determined from simultaneous observations of urinary excretion of radioiodine did not disclose any significant difference. The latter method therefore appears to be an appropriate means of estimating the disappearance rate of radioiodine from blood.

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INDUCED INSULIN RESISTANCE IN THE RABBIT¹

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A high degree of resistance to insulin occasionally develops in patients receiving repeated injections of insulin. This occurs most frequently in patients with diabetes, but non-diabetic individuals receiving insulin shock therapy for psychiatric disorders may also develop insulin resistance unassociated with any other demonstrable disturbance in carbohydrate metabolism. An immune mechanism has been repeatedly suggested as one underlying cause, a view for which strong evidence was obtained in the study of a case of insulin resistance in this laboratory (1-3). Resistance developed during the administration of insulin at the end of three insulin-free periods of three to five months, suggesting an immune response to the injected insulin. Furthermore, the resistance appeared to be species-specific as indicated by absence of resistance to human insulin at a time when resistance to commercial (beef and pork) insulin was clearly present. These observations prompted the study reported herein.

MATERIALS AND METHODS

Twenty-four adult rabbits of various breeds were injected subcutaneously according to a schedule similar to that shown in Table I for the fifth animal in the series. The injection mixture was made as follows: 1) Two parts of an insulin solution containing 250 u/ml. with 0.5% phenol, 2) Three parts of Falba² and 3) Five parts of mineral oil containing 5 mg. dry, heat-killed *M. butyricum* per ml.³ The insulin and Falba were thoroughly mixed with an electric stirrer and the mineral oil containing the acid-fast organisms was then added and likewise thoroughly mixed. Each milliliter of the final mixture contained 50 u of insulin. Preliminary tests showed that the injection of more than 50 u into rabbits often caused severe hypoglycemic symptoms or death. Masses of various sizes and consistencies appeared at the injected sites and in some instances drained

purulent material. Before receiving the insulin-adjuvant mixture, some animals were injected intravenously in the fasting state with 0.1 u crystalline insulin per kilogram in order to test the responsiveness to insulin. In every instance in which this was done, a definite but brief fall in blood sugar occurred. Blood sugar determinations using 0.2 ml. of blood were done with the micro-method described by Nelson (4), using the Folin method of precipitation (5). Blood was obtained from the ear veins with a No. 26 hypodermic needle and a dry tuberculin syringe. The animals were not tied down or otherwise forcibly held. Struggling could be avoided in most instances by infiltrating the area around the vein with novocaine. Insulin tolerance tests with 0.1 u/kg. were repeated at intervals of five to ten weeks and further injections of the insulin-adjuvant mixture were given according to a schedule similar to that shown in Table I. The human insulin used in these experiments was prepared in the summer of 1947 from human pancreas obtained from two to 27 hours post-mortem with a method modified after Somogyi (6). The material was sterilized by passage through a Seitz filter and phenol was added to make a final concentration of 0.5%. Assay in mice and rabbits indicated that each milliliter contained 24 ± 2 u/ml. The details of the preparation and assay of the human insulin are given elsewhere (7).

RESULTS

Of the 24 animals injected with the insulin-adjuvant mixture, four became insulin-resistant. One of these animals, the first to become resistant, has been studied in detail and forms the subject of this report. One of the other resistant animals died of causes apparently unrelated to the experimental procedure, and the remaining two appear to be in good health. Of the 20 rabbits which failed to become resistant, nine died within six months of starting immunization. Ten animals have received injections of the adjuvant mixture for eight months or more without becoming resistant.

The fifth rabbit in our series developed clear-cut resistance to insulin three months after injections of the insulin-adjuvant mixture were begun. All the procedures carried out in this animal are shown in Table I. The number of tests performed was limited by the tendency of the ear

¹ This work was supported by a grant-in-aid from the U. S. Public Health Service.

² Pfaltz and Bauer, Inc., Empire State Building, New York.

³ The authors are indebted to Dr. Jules Freund for advice and for supplying the strain of *M. butyricum*.

veins to become thrombosed after continued use, though care was taken to avoid this. Some of the insulin tolerance tests shown in Table I are irregular, as for example those done on 10/15/47, 11/4/47 and 11/19/47. These irregularities may

be due to undetected errors in technic but the data clearly indicate the development of a high degree of resistance to crystalline insulin. In this animal, a dose of 0.1 u/kg. crystalline insulin intravenously had caused a fall of 20 mg./100 ml.

TABLE I
Procedures done in the insulin-resistant rabbit

Date	Weight	Procedure	Results of insulin tolerance tests. Blood glucose mg./100 ml.							
			FBS	Minutes after injection of insulin						
				15	30	45	60	90	120	180
2/5/47	kg. 2.3	Crystalline insulin 0.1 u/kg. I.V.	118	100	98		113		113	
5/29/47- 6/30/47		Four subcutaneous injections of adjuvant mixture containing 25 u crystalline insulin in 0.5 ml.								
7/9/47	3.15	Crystalline insulin 0.1 u/kg. I.V.	93	79	76		89		85	
7/30/47	3.4	1 ml. adjuvant mixture subcutaneously containing 50 u crystalline insulin								
9/17/47	3.6	Crystalline insulin 0.1 u/kg. I.V.	114	108	117		129		95	101
9/19/47	3.6	Crystalline insulin 0.1 u/kg. I.V.	76	85	76		89			97
9/23/47	3.6	Crystalline insulin 0.4 u/kg. I.V.	108	97	87		103		101	101
10/8/47		1 ml. adjuvant mixture subcutaneously containing 50 u crystalline insulin								
10/15/47		Crystalline insulin 0.8 u/kg. I.V.	80	109	109		85		105	93
10/17/47		Crystalline insulin 1.6 u/kg. I.V.	105*		117		117			
10/20/47		Crystalline insulin 4.4 u/kg. I.V.	91*		64		76		109	
10/24/47		1 ml. adjuvant mixture subcutaneously containing 50 u crystalline insulin								
10/29/47		Human insulin 0.32 u/kg. I.V.	81		42		84	87	97	
10/31/47		Human insulin 0.32 u/kg. I.V.	90	36	60	74	85	90	97	109
11/4/47		Crystalline insulin 2.5 u/kg. I.V.	79	108	71	63	100	103	104	111
11/19/47		Crystalline insulin 0.32 u/kg. I.V.	144	99	101	104	105	106	103	104
1/6/48	4.08	Crystalline insulin 0.5 u/kg. I.V.	96	94	84	84	85	94	94	
1/8/48	4.08	Cysteine-inactivated insulin 15 u/kg. I.V. followed in 20 minutes by crystalline insulin 0.5 u/kg. I.V. The inactivated insulin caused a rise in blood sugar from 87 mg./100 ml. to the fasting level shown.	104	59	54	67	66	72	80	
1/12/48	4.08	Cysteine-inactivated insulin 15 u/kg. I.V. This caused a rise in blood sugar from 80 mg./100 ml. to the fasting level shown. No active insulin given.	132	111	100	101	99		91	
2/26/48	3.96	Crystalline insulin 0.5 u/kg.	88	92	95	97	98	102	82	
3/19/48		Crystalline insulin 2.5 u/kg.	80		93		92	92		

* Animal not fasted.

INTRAVENOUS INSULIN TOLERANCE TESTS IN THE RESISTANT RABBIT
AND IN NORMAL AND UNSUCCESSFULLY IMMUNIZED RABBITS

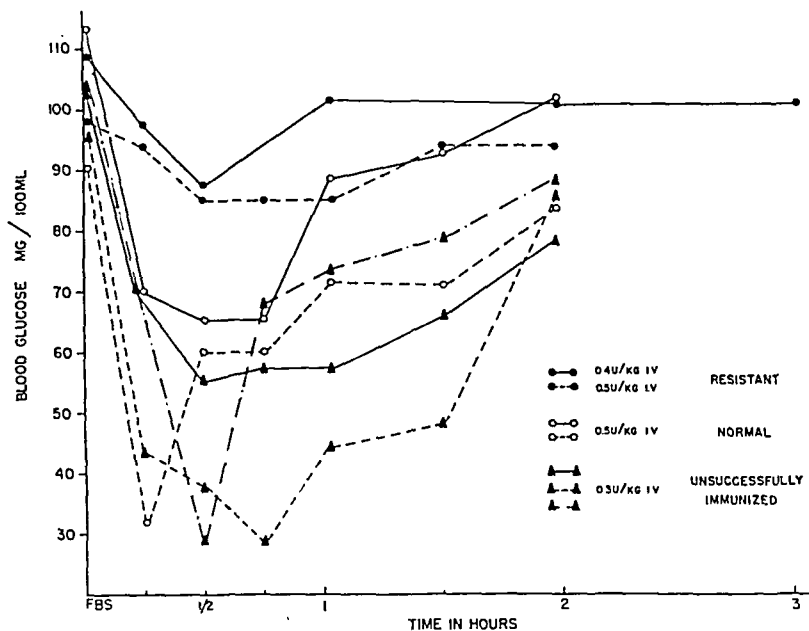


FIG. 1

The dates on which the insulin tolerance tests in the resistant rabbit were done were as follows:

0.4 u/kg. I. V. 9/23/47. This test is also shown in Figure 3.

0.5 u/kg. I. V. 1/6/48. This test is also shown in Figures 3 and 5.

in the blood sugar before any injections of the adjuvant mixture were given and a similar fall followed the same dose of insulin six weeks after these injections were begun. After three or more months, four or five times this dose (0.4 u/kg. and 0.5 u/kg.) given intravenously caused a fall of 21 mg./100 ml. or less as shown in the two upper curves of Figure 1. The blood sugars of two normal rabbits receiving 0.5 u/kg. fell 40 mg./100 ml. and 60 mg./100 ml., respectively, and in two other animals, which received the adjuvant mixture but failed to become resistant, marked falls in blood sugar followed the injection of 0.3 u/kg. Further tests done in the resistant animal are shown in Figure 2 where it may be seen that a large dose of insulin (4.4 u/kg.) was required to produce a moderate fall in blood sugar. Two of the tests shown in Figure 2 were done without fasting in order to decrease the risk of killing the animal in hypoglycemic shock, a precaution which was probably unnecessary. The curve obtained in the fasting state with 2.5 u/kg. was done at a later date than the others and we

INTRAVENOUS TOLERANCE TESTS WITH LARGE DOSES OF COMMERCIAL
INSULIN AFTER DEVELOPMENT OF RESISTANCE

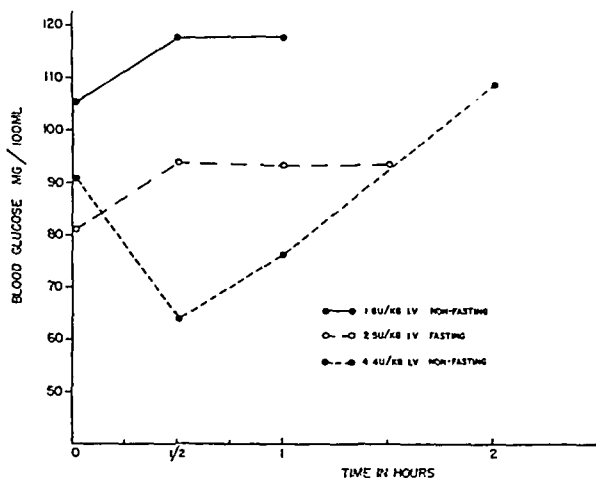


FIG. 2

The dates on which the insulin tolerance tests were done were as follows:

1.6 u/kg. I. V. non-fasting 10/17/47.

2.5 u/kg. I. V. fasting 3/19/48.

4.4 u/kg. I. V. non-fasting 10/20/47.

have reason to believe, on the basis of protection studies in mice (8) with the animal's serum, that a higher degree of resistance was present at this time. These studies will be reported later.

The condition of the site at which the adjuvant mixture was injected did not appear to influence the animals' response to insulin as judged by the normal insulin tolerance curves obtained in animals with fluctuant masses or draining sinuses. Furthermore, only one of the four animals that became resistant developed any significant inflammation at the injected site, and even in this subject resistance to insulin was clearly demonstrable when the inflammation had subsided.

In none of the tolerance tests done in this animal were there any symptoms suggesting a hypoglycemic reaction or any allergic manifestations such as scratching, dyspnea, or collapse. The endermal injection of 0.02 ml. of crystalline insulin in a concentration of 4 u/ml. on two occasions caused no immediate or delayed local reaction.

The repeated finding of a normal fasting blood sugar indicated that the animal had no resistance to his own insulin. Furthermore, the animal appeared to be in good health and gained weight steadily. An intravenous glucose tolerance curve with 0.5 gm. glucose/kg. gave the following result suggesting normal carbohydrate metabolism. FBS 90 mg./100 ml.; 30 minutes, 116 mg./100 ml.; one hour, 88 mg./100 ml.; two hours, 88 mg./100 ml.; three hours, 88 mg./100 ml. In view of the presence of resistance to commercial insulin, these results indicated the presence of a species-specific immunity to beef and pork, but not to rabbit insulin.

Further evidence for the species specificity of the resistance was obtained in tests with human insulin. Curves are shown in Figure 3 where approximately 0.3 u/kg. of human insulin caused falls in blood sugar of 39 mg./100 ml. and 54 mg./100 ml. results which were similar to those obtained in normal animals receiving either human or crystalline insulin in this dosage. As

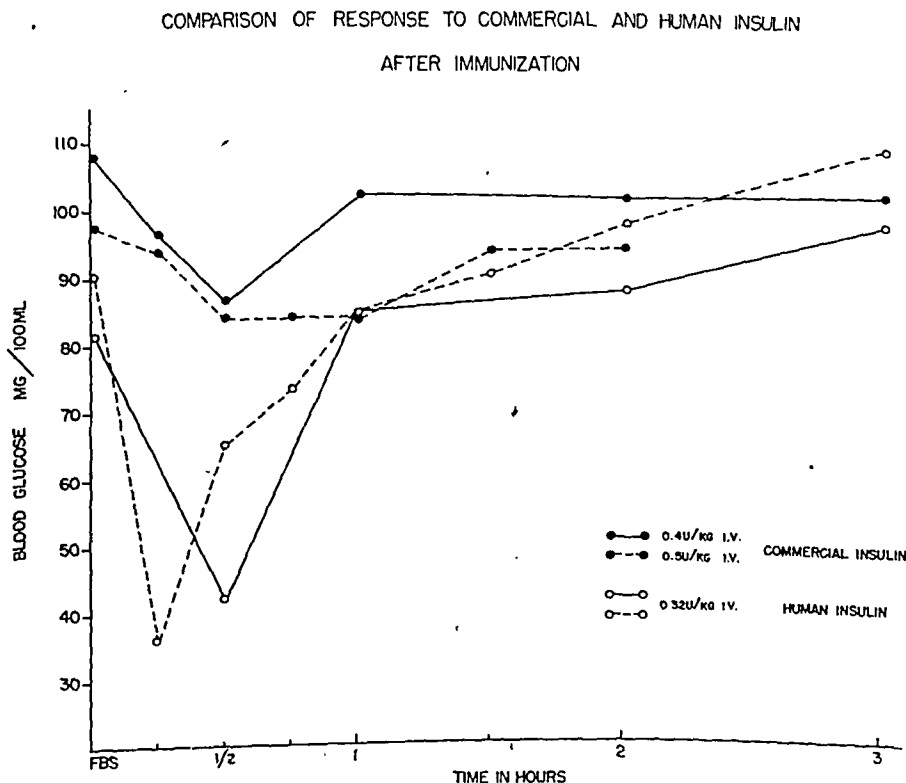


FIG. 3

The dates on which the insulin tolerance tests were done were as follows:

- 0.4 u/kg. I. V. commercial insulin 9/23/47.
- 0.5 u/kg. I. V. commercial insulin 1/6/48.
- 0.32 u/kg. I. V. human insulin (solid line) 10/29/47.
- 0.32 u/kg. I. V. human insulin (dotted line) 10/31/47.

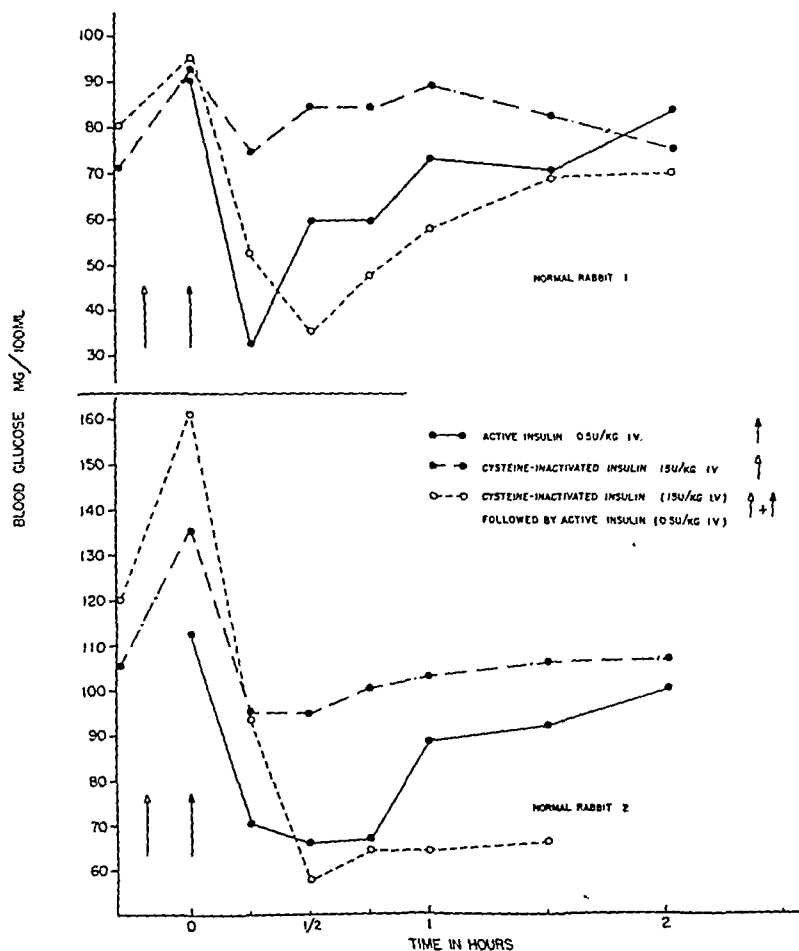
TESTS WITH ACTIVE INSULIN, CYSTEINE-INACTIVATED INSULIN AND THE
COMBINATION OF THE TWO IN NORMAL RABBITS

FIG. 4

described above, much smaller falls in blood sugar followed the injection of 0.4 u/kg. and 0.5 u/kg. of commercial insulin. The tests with human insulin were done in the period between the two tests with commercial insulin and therefore it is unlikely that the responsiveness to human insulin was due merely to the loss of a resistance which was not species-specific.

A final experiment was based on the assumption that the resistant animal's antibody was directed against antigenic determinants on the insulin molecule which were distinct from the group or groups to which the hormone owes its specific effect on carbohydrate metabolism. Accordingly, if the insulin molecule were shorn of its active group, antibody might still combine with it and the injection of a large amount of such

insulin into the resistant animal, by combining with the animal's antibody, might then bring about temporary susceptibility to the action of a relatively small dose of active insulin.

To this end, 400 u of crystalline insulin were inactivated under reduced oxygen tension with 167 mg. cysteine HCl in the presence of sufficient NaOH to bring the pH to approximately 7.0 (9). After the addition of phenol to make a final concentration of 0.5%, the solution contained the equivalent of 27 u/ml. During the procedure and upon exposure to room air the cysteine in this mixture slowly became oxidized to cystine forming a precipitate which was resuspended each time the solution was used. When tested in fasting mice, 0.5 ml. of a 1:5 dilution failed to induce any symptoms at 35° C., indicating that

INFLUENCE OF CYSTEINE-INACTIVATED INSULIN ON THE SUSCEPTIBILITY OF THE
INSULIN-RESISTANT RABBIT TO ACTIVE INSULIN

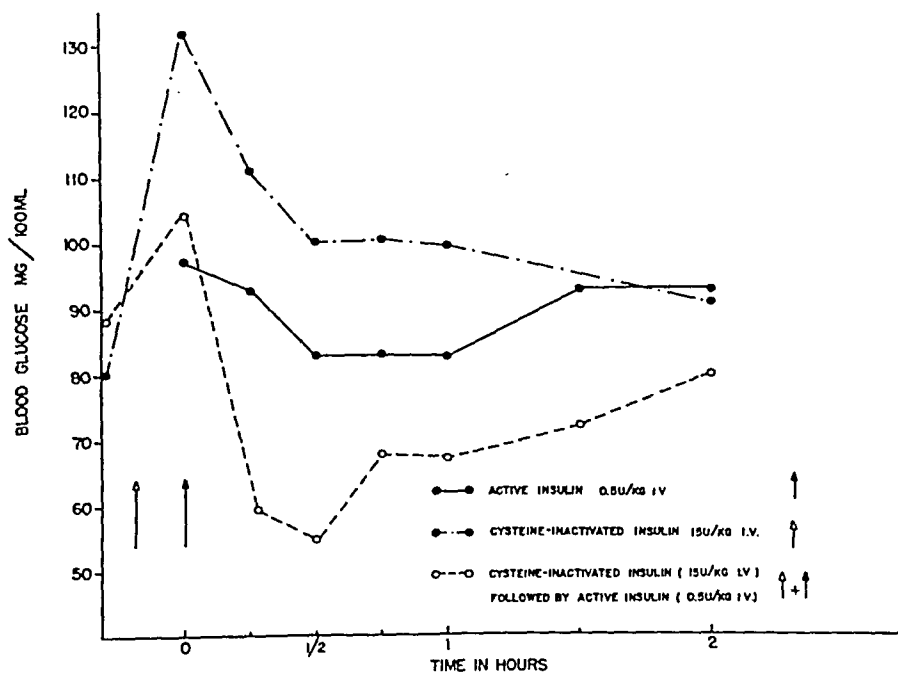


FIG. 5

The dates on which the insulin tolerance tests were done were as follows:

Active insulin 0.5 u/kg. I. V. 1/6/48.

Cysteine-inactivated insulin 15 u/kg. I. V. 1/12/48.

Cysteine-inactivated insulin followed by active insulin 1/8/48.

the insulin had been at least 99% inactivated.

The experiments with cysteine-inactivated insulin are shown in Figures 4 and 5. Each experiment consisted of three blood sugar curves done with cysteine-inactivated insulin alone, active insulin alone and cysteine-inactivated insulin followed in 20 minutes by active insulin. All injections were made intravenously. In each instance, the injection of the inactivated insulin caused a temporary rise in blood sugar which may have been due entirely or in part to the cysteine present. In the normal animals, the hypoglycemic effect of active insulin given after cysteine-inactivated insulin was like that of active insulin alone. A strikingly similar result was obtained in the resistant animal though active insulin alone caused only a slight fall in blood sugar. Thus, two agents—one of which lacked blood sugar lowering activity and indeed tended to raise the blood sugar, and the other having only a feeble blood sugar lowering effect in this animal—combined to produce a pronounced fall

in blood sugar. These results indicate that the inactivated hormone combined with the animal's antibody, leaving the active insulin free to lower the blood sugar.

DISCUSSION

The experimental production of a species-specific resistance to insulin in the rabbit on an immunological basis is evidence for the antigenicity of insulin and gives further support to the view that some instances of resistance to insulin in man have an immune basis. The element of species specificity explains the coexistence of resistance to exogenous insulin on the one hand and undisturbed utilization of endogenous insulin on the other. This appears to be the case in the resistant rabbit reported herein as well as in certain instances of insulin resistance which have been observed among non-diabetic patients receiving insulin-shock therapy.

It is difficult on the basis of our observations to arrive at a conclusion regarding the antigenic-

ity of insulin in the adjuvant mixture used, owing to the high mortality of our animals. It can be safely stated, however, that with the method used only a small proportion of animals will develop resistance within three months of the time immunization is begun. The possibility remains, however, that all animals might become resistant if immunization were sufficiently prolonged. The failure of other workers to produce unequivocal resistance to insulin on an immunological basis in experimental animals may have been due to the omission of adjuvants and perhaps the use of an insufficiently sensitive test for the detection of resistance. In their paper describing the successful production of complement-fixing antibodies to insulin in rabbits receiving large intravenous and subcutaneous injections of insulin, Wasserman *et al.* state (10) that no resistance to insulin developed. However, the method used in attempting to show resistance was not described. The relationship between complement-fixing antibodies, precipitins and susceptibility to anaphylaxis on the one hand and resistance to insulin on the other will require further investigation.

Earlier studies in this laboratory (1, 2) as well as those reported herein are in conflict with the claim made by Wasserman and Mirsky (11) that insulins from various animal sources are immunologically identical. Indeed the observations in our insulin-resistant animal can hardly be explained other than by assuming different immunologic specificities for rabbit and human insulins on the one hand and beef and pork on the other. There is one possible, but unlikely, explanation for the results obtained by us, which retains the concept of the immunologic identity of all insulins. The assumption can be made that the modern method for the commercial extraction and purification of insulin alters the molecule so as to confer upon it a new immunologic specificity. According to this hypothesis, the resistant animal's antibody is directed against insulin modified by the method currently used in the preparation of commercial insulin and would fail to combine with native insulin (endogenous rabbit insulin) or human insulin extracted by the older method used by us.

The experiment with cysteine-inactivated insulin suggests that the antigenic specificity of the insulin molecule is little altered by inactivation,

a result which is in accord with conclusions reached by Wasserman *et al.* (10). This experiment also indicates that the total available antibody in the resistant animal was not in excess of an amount which would neutralize 60 u of insulin (15 u/kg.). The estimated degree of resistance in this animal may therefore be less than that seen in some instances of human resistance to insulin which may require more than 5000 u in 24 hours (70 u/kg.). However, regeneration of antibody during the 24-hour period may be one factor contributing to the large insulin requirement in such cases.

SUMMARY

Resistance to the hypoglycemic action of commercial crystalline (beef and pork) insulin was induced in the rabbit by the repeated subcutaneous injection of crystalline insulin incorporated in an adjuvant mixture. The resistance to insulin was species-specific, as indicated by both the animal's failure to develop hyperglycemia and the response to human insulin, and appeared to be due to the development of an antibody for insulin. Evidence is presented which indicates that insulin treated with cysteine so as to destroy its hypoglycemic activity retains its capacity to combine with antibody.

We take pleasure in expressing our indebtedness to Miss Jean Buckley for technical assistance.

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RELATIVE MEASUREMENT *IN VIVO* OF ACCUMULATION OF RADIOIODINE BY THE HUMAN THYROID GLAND: COMPARISON WITH RADIOACTIVITY IN PERIPHERAL TISSUES¹

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The gamma rays produced by radioiodine permit its direct measurement *in vivo*, and particularly in the thyroid gland, both of the human subject and of animals, with little or no disturbance of existing equilibriums, provided, that is, that the possibility of a biologic effect of radioiodine due to its radiation per se is disregarded. Full realization of the possibilities of this quantitative method of measurement has been thwarted by difficulties of interpreting the observations as well as by numerous technical difficulties which interfere with accuracy in the absolute measurement of radioiodine *in vivo*. These difficulties include geometric problems arising from anatomic variations in the thyroid and surrounding structures and the problem of evaluating the degree of absorption and scattering of radiation that occurs. However, in spite of the foregoing considerations, relative measurements over the thyroid, expressed simply in counts per second, have yielded useful data.

Hamilton and Soley (1) studied the collection of I^{131} by the thyroid in 17 subjects after administration of the radioiodine with 14 mg. of inert iodide as carrier. Data from normal individuals were recorded in smooth curves which reached a plateau about two days after administration of the dose and thereafter remained flat. Curves of collection for patients with nontoxic goiters resembled those of normal persons but larger quantities of radioiodine were collected. Thyrotoxic glands had a maximal uptake of I^{131} in one to four hours and the uptake then fell rapidly in 24 hours to one half to one fifth the maximal value.

A nontoxic goiter in a child who had hypothyroidism showed a curve like that produced by the thyrotoxic glands and the thyroid glands of four children having hypothyroidism without goiter collected only a small amount of radioiodine. Later Hamilton (2) reported that, when 0.1 μ g. instead of 14 mg. of iodide was used as carrier, the maximal uptake in hyperthyroid glands was several times greater and the rapid loss of radioiodine from the thyroid previously observed did not occur. In 1943, Hamilton and his associates (3) reported that the thyroids of two children who had hypothyroidism and goiters collected and retained relatively large amounts of radioiodine. Hertz (4) in 1941 made *in vivo* measurements over the thyroid region and found that the uptake of radioiodine in untreated hyperplastic thyroid glands approached 100 per cent.

Quimby and McCune (5) in 1947 made *in vivo* measurements of radioiodine in children. The mean collection by normal subjects was 12 per cent of the dose. The thyroids of hyperthyroid patients collected substantially more than this whereas hypofunctioning glands collected less than 1 per cent of the dose. Stanley and Astwood (6) in 1947 administered I^{131} in doses of 100 microcuries without carriers to normal subjects for the purpose of assaying antithyroid compounds. When the counts per second recorded over the thyroid were plotted against the square root of time, the observations could be fitted to a straight line, from which was calculated an arbitrary numerical value which they called an "accumulation gradient." The value of this gradient in euthyroid subjects varied from 1.4 to 36.4 with a mean value of 9.4.

Keating, Power, Berkson and Haines (7) have described studies on the excretion of radioiodine in the urine of human subjects. They indicated

¹ Abridgment of thesis submitted by Dr. Luellen to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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that neither the proportion of a dose of radioiodine eventually excreted in the urine (the renal fraction) nor the rate constant estimated from the curve of urinary excretion could be regarded as direct or quantitative measures of the function of the kidney with regard to excretion of iodine. The former they felt to be a relative quantity depending on the capacity of the thyroid and of other tissues to dispose of iodine as well as of the kidney to excrete it, and the latter they inferred to be the rate of disappearance of radioiodine from blood. McConahey and co-workers (8) confirmed the latter assumption by showing that no significant difference existed between the rate of disappearance of radioiodine from blood, as determined directly by examination of the blood itself, and the rate of disappearance obtained from simultaneous observations on urine. The measure of renal excretion of radioiodine was shown to be the product of the renal fraction and the rate of disappearance of radioiodine from blood.

Keating and associates inferred from their analysis of urinary excretion that the same considerations would apply to the accumulation of

radioiodine by the thyroid; namely, that both the proportion of a dose of radioiodine eventually collected by the thyroid, and the rate constant derived from a curve of radioiodine accumulation by the thyroid, would prove to be *indirect* and *relative* rather than *direct* and *quantitative* measures of the function of the thyroid with respect to accumulation of iodine.

It was the purpose of our investigation to apply the foregoing considerations directly to *in vivo* measurement of radioiodine in the thyroid of human beings and, particularly, to compare the kinetic behavior of radioiodine in the thyroid itself with observations *in vivo* on peripheral tissues, the concentration of radioiodine in the blood and the urinary excretion of this substance.

METHODS AND MATERIALS

The Geiger counter tubes³ used contained mica windows of approximately 3 mg. per square centimeter or about 0.01 mm. thick and 3.0 cm. in diameter. Counts were recorded by means of a scaling circuit with a scale of 256. The principle of a shield as described by Seidlin *et al.* (9) was used; this consisted of a solid lead cylinder 5.2 cm. thick and 11.5 cm. in diameter to interpose be-

³ Manufactured by the Radiation Counter Laboratories.

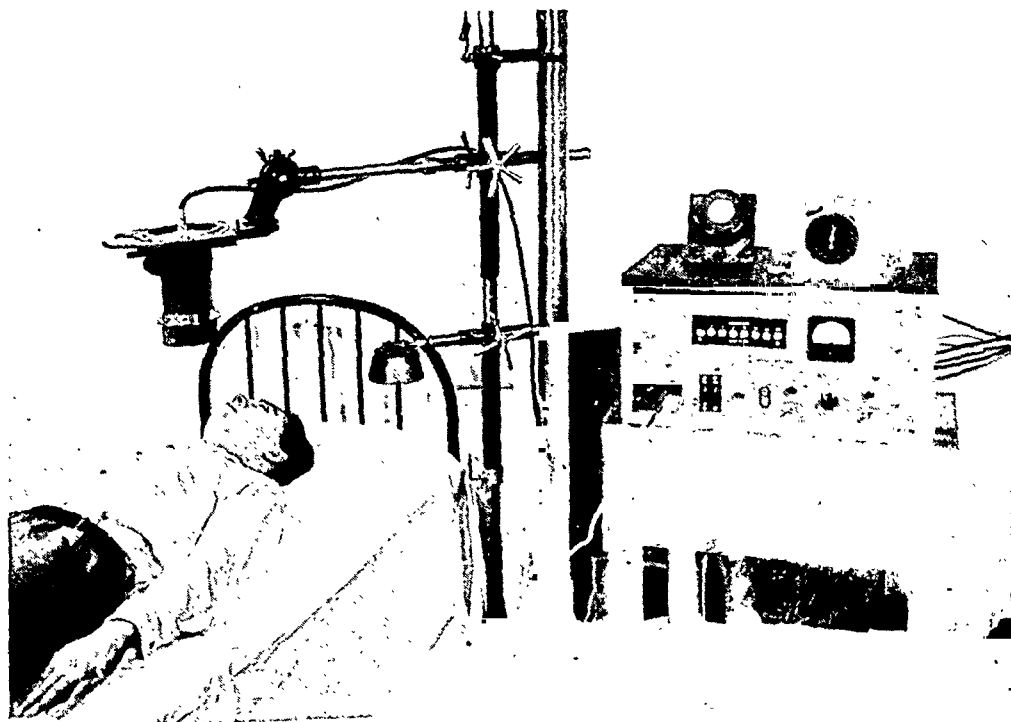


FIG. 1. APPARATUS FOR *IN VIVO* MEASUREMENT OF RADIOIODINE IN THE THYROID WITH A GEIGER COUNTER

Note shielded counter tube mounted on a roentgen-ray tube standard, movable shield to be interposed between the thyroid and the window of the counter, scaling circuit and recording clock.

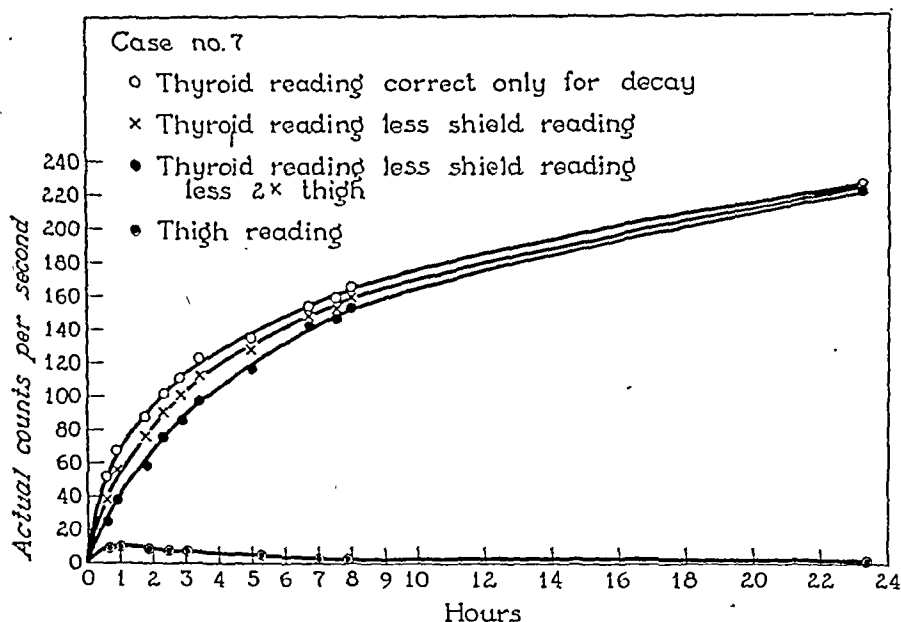


FIG. 2. RADIOIODINE ACCUMULATION CURVES IN A CASE OF ADENOMATOUS GOITER WITHOUT HYPERTHYROIDISM ILLUSTRATING THE MAGNITUDE OF THE CORRECTION FOR RADIATION FROM THE BODY AND FROM THE TISSUES OF THE NECK

tween the thyroid gland and the counting tube, which when in place blocked out more than 99.5 per cent of the direct gamma rays from the thyroid gland. To allow enough room to interpose the lead shield and to have the tube far enough away to receive rays from the entire gland a standard distance of 10 cm. above the thyroid and thigh was chosen at which to place the counter tube. Readings taken with the shield were subtracted from readings without the shield. This corrected for background radiation from the entire body.

The counter tube was kept in an assembly attached to a standard so that it could be raised, lowered or turned in any direction (Figure 1). The assembly consisted of a lead cylinder with a wall 16 mm. in thickness surrounding the counting tube. Over the window of the tube at the lower end of the assembly was a 2.7 mm. brass filter. (Low counts were increased by using instead a filter consisting of 0.2 mm. copper with 0.04 mm. lead.) A pad was placed under the patient's neck so that the arch of the neck would remain constant. Adhesive tape was placed over the center of the thyroid. The tube assembly was centered over a cross on the center of the adhesive tape by using a thin brass plate, which exactly fitted the lower end of the tube assembly, and the center of which had a small chain attached to it to act as a plumb line. Readings were taken with and without the shield interposed above the thyroid and then with and without the shield above the thigh and 10 cm. cephalad to the patella. Whenever there was considerable discrepancy between any two readings, a third or fourth reading was made. The measurements over the thigh were made primarily to correct for the radiation due to radioiodine in the extracellular fluids of the neck. Including the time for setting the tube, the time for one

such series as just described which led to a single point on a graph required about 30 minutes.

The I^{131} was given to fasting subjects in 100 μ g. of sodium iodide as carrier. Tracer doses of I^{131} consisted of about 100 to 1,700 microcuries and therapeutic doses of more than 2,500 microcuries. The neck was palpated and the center of the thyroid gland (if palpable) was marked with adhesive tape on which a cross was marked. With the patient lying face upward on a cot, readings were taken with the counter tube set 10 cm. above the thyroid. Readings were made every 30 to 90 minutes for eight hours and daily thereafter.

In a series of seven patients who apparently did not concentrate radioiodine in their thyroid glands, it was found that the average ratio of radioactivity over the neck, compared to that over the thigh after the ratio became relatively constant, was 2.3 with a range of 2.1 to 2.6. Since in each given case the neck-to-thigh ratio would only be approximate, it was arbitrarily decided to subtract two times the reading obtained from the thigh to correct for the radioiodine in the blood and extracellular fluid in the neck. Figure 2 illustrates the magnitude of the corrections which were made for body radiation and radiation of the extrathyroid part of the neck in an average euthyroid subject. The top curve is that which was obtained when neither of these corrections was made. The second curve from the top shows the effect of correction for body radiation (subtraction of the readings over the thyroid area with the shield interposed). The third curve from the top is the one which was used routinely and was obtained by subtracting twice the values for thigh from the values plotted in the second curve from the top. In cases in which the uptake of radioiodine in the thyroid gland was larger, such cor-

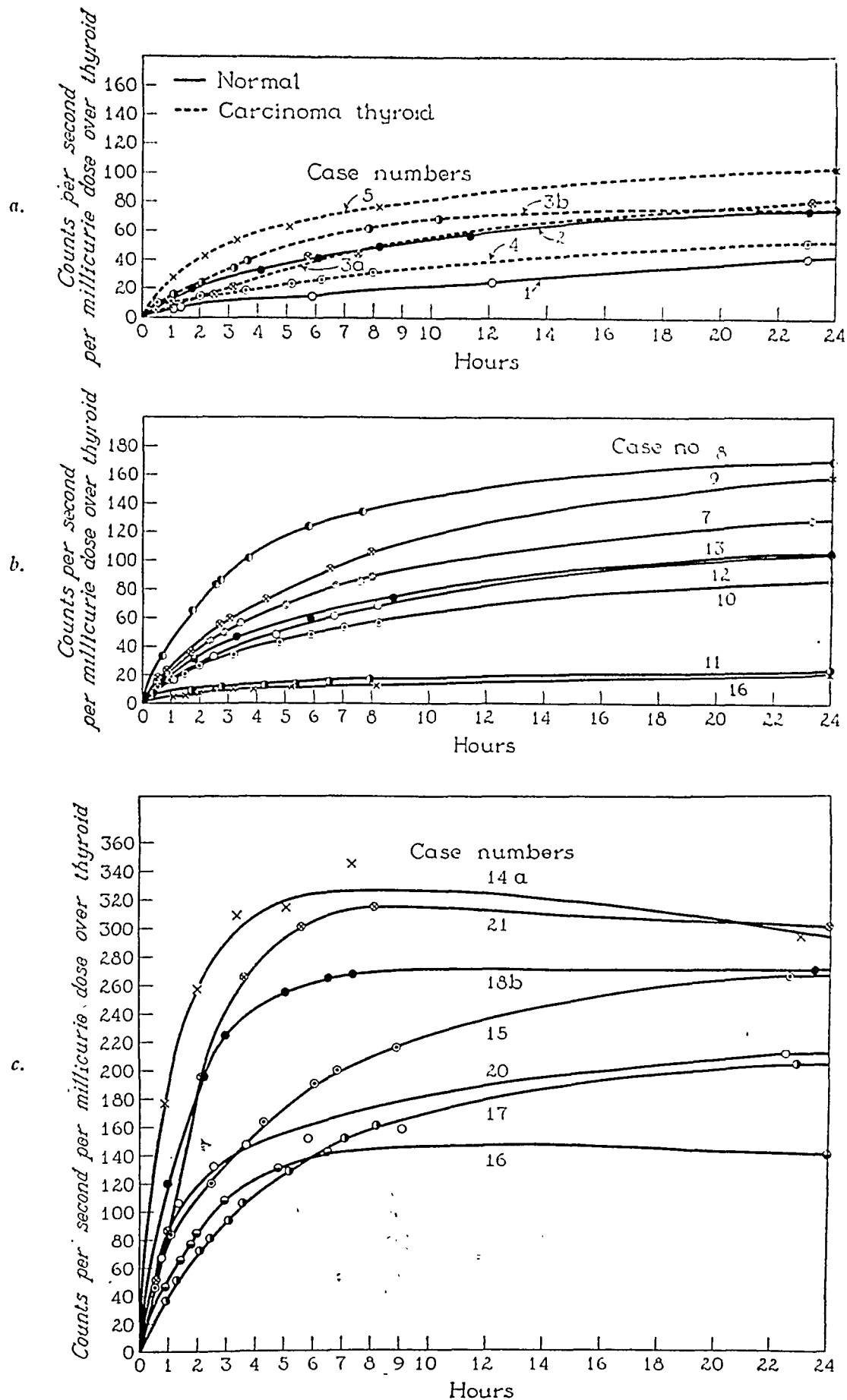


FIG. 3. INDIVIDUAL CURVES OF ACCUMULATION OF IODINE IN THE THYROID

a. For normal volunteers and euthyroid individuals with carcinoma of the thyroid. b. For individuals with adenomatous goiters without hyperthyroidism. Some of these appear to collect substantially more than other euthyroid patients. c. For individuals with hyperthyroidism who received tracer doses. Curves constructed from therapeutic doses did not differ significantly.

rections made even less difference than in the case illustrated.

For a patient receiving a large dose of I^{131} for therapeutic purposes, the counting tube was not able to record all the counts. It was necessary in such situations to use a lead filter in addition to the brass filter over the window of the tube. The sensitivity of the tube was checked against a radium standard. Corrections were made for the resolving time of the counting tube, the decay of the I^{131} , and for the various filters used.

Fourteen studies were made of 13 euthyroid subjects including two normal volunteers, three patients having carcinoma of the thyroid of low grade of malignancy and eight patients having adenomatous goiters without hyperthyroidism. Observations in one case were previously reported by Keating and his co-workers. Fifteen studies were made on ten patients who had hyperthyroidism. Six studies were made on six myxedematous patients. Studies of the blood and urine were carried out as described in previous papers (7, 8).

RESULTS

Figure 3a shows individual iodine-accumulation curves of normal volunteers, and of euthyroid patients with thyroid carcinoma of low grade of malignancy; Figure 3b shows the individual curves of euthyroid patients with adenomatous goiters, and Figure 3c the individual curves of patients with exophthalmic goiters receiving tracer doses. Curves obtained in five additional cases of exophthalmic goiter after therapeutic doses and

curves obtained in six cases of myxedema are not shown individually but are summarized in Figure 4. In order to place all subjects on a comparable basis as far as dosage was concerned, observations of radioactivity were expressed as counts per second per millicurie of dosage. That is, the counts per second as observed were divided by the millicuries of dosage before plotting. When different filters were used, a similar adjustment was made for the effect of the filter. Each count observed at 10 cm. was equal approximately to 2.5 microcuries. Such adjustments for dosage and filter are valid as far as they go but comparison from case to case even with these corrections is at best a rough approximation since it takes no account of the varying shapes of the thyroid and the varying locations in the necks in different individuals.

The curves were all exponential. The curves of the normal individuals and of euthyroid patients with carcinomas or adenomas without hyperthyroidism reached their peak value in about 24 to 48 hours and, thereafter, diminished very slowly. The curves of hyperthyroid patients, whether obtained after tracer or therapeutic doses, rose more steeply and to higher peaks and, thereafter, decreased more rapidly than the curves of euthyroid persons. It made no apparent differ-

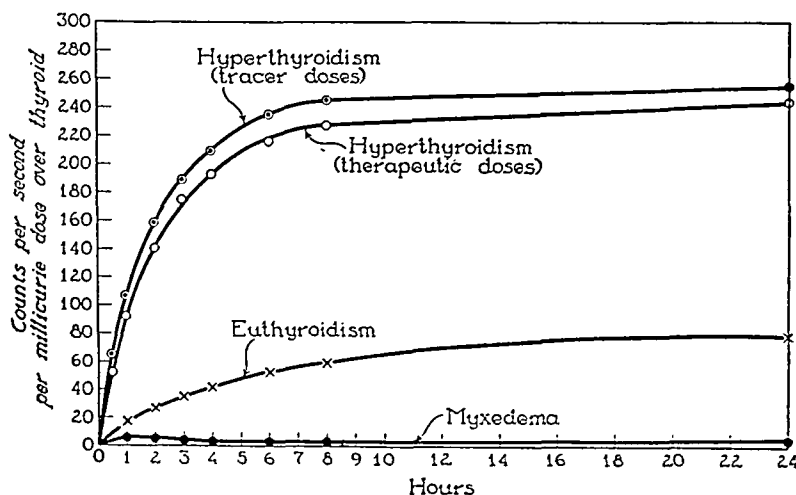


FIG. 4. COMPOSITE ACCUMULATION CURVES CONSTRUCTED FROM THE MEAN OBSERVATIONS FOR EACH POINT IN TIME FOR EACH GROUP

The curve for myxedema represents six cases, in none of which did the thyroids retain significant quantities of radioiodine. The curve for therapeutic doses in hyperthyroidism does not differ significantly from the curve for tracer doses.

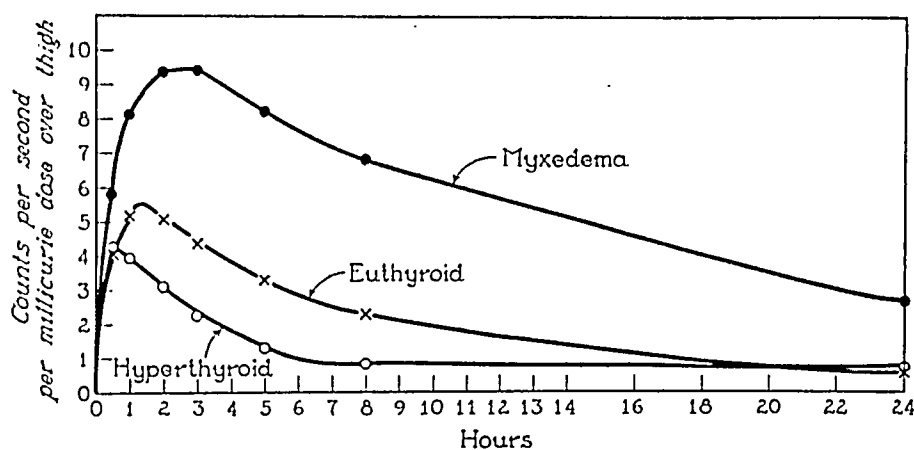


FIG. 5. COMPOSITE CURVES OF RADIOACTIVITY OVER THE THIGH CONSTRUCTED FROM THE MEAN OBSERVATIONS FOR EACH POINT IN TIME FOR EACH GROUP

These curves correspond in form to those obtained by McConahey and associates by analysis of blood samples.

ence in the shape of the curves or in their apparent magnitude expressed as counts per second per millicurie whether tracer or therapeutic doses were measured. There was some overlapping between the lowest curves for hyperthyroid patients and the highest curves for euthyroid subjects. The curves obtained in cases of myxedema showed little or no accumulation of radioiodine in the thyroid.

Figure 4 shows the composite curves for each of the four types of condition studied computed from the means of the observations versus time: (1) euthyroidism, all cases, (2) hyperthyroidism with tracer doses, (3) hyperthyroidism with therapeutic doses, and (4) myxedema. The peaks in cases of hyperthyroidism were about three times as great as the peaks in cases of euthyroidism and half of the peak value was reached in less than two hours while the euthyroid curve reached half its peak value in about four hours.

Two patients were studied who clinically were thought to have hyperthyroidism but who failed to collect radioiodine. The neck-to-thigh ratio in each case was approximately 2:1 and in each the radiation over the neck decreased at the same rate as over the thigh. No concentration of radioactivity could be detected in the pelvis or other parts of the body.

One patient was a nervous woman, aged 33 years, whose basal metabolic rates ranged from +23 to +37 per cent. She had taken iodine for a year and had discontinued its use two weeks prior to the tracer study. At operation 6 gm.

representing approximately three fourths of the thyroid was removed. It showed slight parenchymatous hypertrophy. Thyroidectomy did not relieve her symptoms.

The second patient was a woman, aged 38 years, whose basal metabolic rates varied from +18 to +23 per cent. Biopsy of the skin disclosed lupus erythematosus; the spleen was enlarged, and the thyroid was not palpable. A cholecystogram had been made 12 days before radioiodine was given. No improvement followed a therapeutic trial of Lugol's solution. Thyroidectomy was not performed.

In Figure 5 composite curves of observations over the thigh are plotted as counts per second per millicurie of dose against time in hours. The conditions studied were (1) euthyroid state in 11 cases; (2) hyperthyroidism in 11 cases, and (3) myxedema in six cases. The curve for patients who had hyperthyroidism had the lowest peak. This was reached within 30 minutes after observations were begun and values, thereafter, decreased the most rapidly of any of the three groups; that is, it decreased to one half of its peak value in two and a half to three hours. The curve for euthyroid patients reached its peak in one hour, at a higher level, and fell more slowly, reaching its half value in about six hours after the peak was reached. Finally, the curve for myxedematous patients indicated that the peak of radioactivity in the blood and extracellular fluid was not reached until about two to three hours after oral administration of I^{131} . This peak was

the highest of the three, and half of the peak value was reached in about 11 to 13 hours. The counts from the thigh with small tracer doses were small and inaccurate owing to the low level of radioactivity. The similarity between curves of measurements from the thigh and from the blood was most striking.

COMMENT

Observations with a Geiger counter, and in particular observations *in vivo*, are subject to numerous sources of error. These include variations in the sensitivity and stability of the counter tube, a statistical counting error which varies with the counting rate as compared to the background count and in the case of high counts a systematic error resulting from the resolving time of the tube. Such purely physical errors can be corrected or minimized by careful technic, but despite the utmost care such errors place a definite limit on the precision of *in vivo* counting. Other errors arise from a consideration of the varying size and shape of the thyroid and surrounding structures in different individuals, variations in radiation entering the apparatus from other parts

of the body than that being measured and so forth. To a certain extent these too can be minimized but not eliminated by careful technic. It is impossible to express quantitatively the net error in the observations which result from the sum of the foregoing and the efforts to minimize them. Such errors become especially important when an attempt is made to measure radioactivity in some absolute term, but even in the present case, in which radioactivity is measured in counts per second, at various intervals of time, one must regard the observations as having an error of the order of magnitude of perhaps 10 per cent. Minor fluctuations, therefore, might easily occur in the quantity of radioiodine in the thyroid which could not be detected by this method.

Inspection of the curves in Figure 3 indicate that, as shown by other observers, the hyperplastic thyroids of hyperthyroid patients accumulate much larger quantities of radioiodine and do so more rapidly than do the thyroids of euthyroid individuals. It is unfortunate that a larger group of normal persons were not available for comparison. We have been reluctant to perform radioiodine studies on more normal volunteers at the

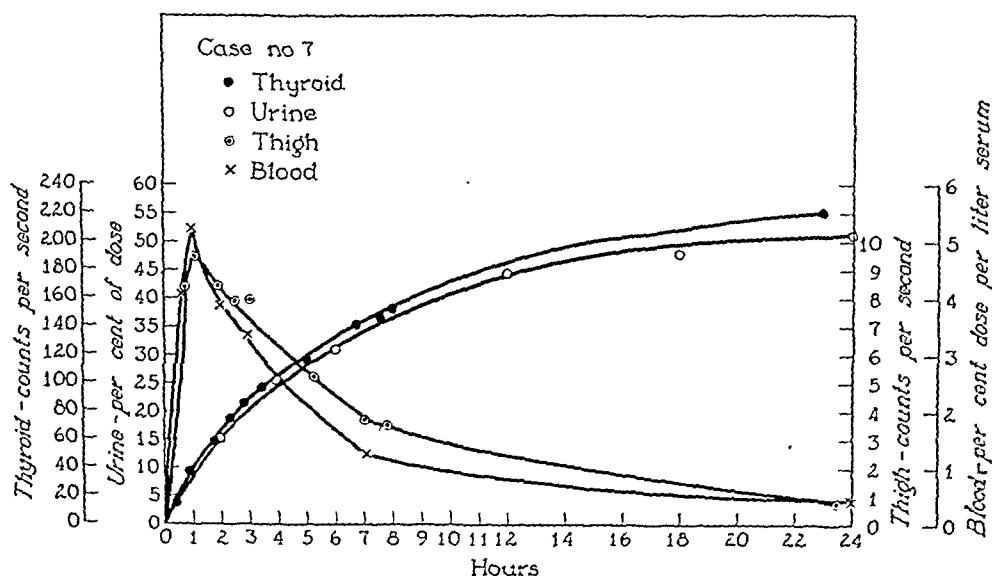


FIG. 6. CURVES DENOTING ACCUMULATION OF RADIOIODINE IN A CASE OF ADENOMATOUS GOITER WITHOUT HYPERTHYROIDISM

Curves were constructed from simultaneous observations made over the thyroid and the thigh and analyses for radioiodine in samples of blood and urine. Note that thyroid and urine curves are similar in form and that blood and thigh curves resemble each other closely. That all four have a common denominator is indicated by the fact that all tend to level off at about the same time.

doses which present technics require because of a growing concern over the quantity of radiation involved. This may be of the order of 12 roentgens equivalent physical (r.e.p.) per day initially and a total integrated dose of the order of 150 r.e.p. when 100 microcuries of I^{131} are given. So far as our observations show, the thyroids of euthyroid individuals with thyroid malignancy of low grade behave in an essentially normal fashion, as do most thyroids of euthyroid individuals with small or moderate adenomatous goiters without hyperthyroidism. For purposes of comparison with hyperthyroid and hypothyroid patients these have been included. In the cases of malignant disease of the thyroid included in this study the malignant tissue, so far as could be determined, did not collect a measurable amount of radioiodine. In a large group of cases of adenomatous goiter without hyperthyroidism in which the urinary excretion of radioiodine was studied, considerably more variation in the behavior of the radioiodine occurred than in other euthyroid groups. A certain proportion of the thyroid glands of these patients, particularly the large nodular goiters, behaved more like hyperthyroid than euthyroid glands. The radioiodine-accumulation curves of the adenomatous goiters without hyperthyroidism included in the present study (Figure 3b) in general resembled those of normal subjects and euthyroid patients with malignant lesions, although several patients retained larger quantities of iodine in their thyroids. One patient in particular (Case 8, Figure 3b) collected more iodine and did so more rapidly than any other euthyroid individual studied. It is probable, therefore, that adenomatous goiters, especially large ones, ought not to be regarded as entirely normal in function even in the absence of clinical hyperthyroidism.

The form of the curve of accumulation of iodine in the thyroid appears on inspection to be exponential and resembles closely the curves constructed from observations of urinary excretion in a previous paper. Furthermore, the form of the curve of radioactivity over the thigh corresponds closely to that constructed from determinations of radioiodine in blood serum. Figure 6 illustrates the relationships of all four types of observations in a case of adenomatous goiter without hyperthyroidism. The existence of a rate constant

common to all four curves can be inferred from the fact that they all tend to level off at about the same time. It is of interest, therefore, to analyze these curves in the manner employed by Keating, Power, Berkson and Haines (7) for the study of urinary excretion of radioiodine.

ANALYSIS OF CURVES

Observations of the quantity of radioiodine in the thyroid can be fitted with an exponential function of the form

$$Q_{\text{thyroid}} = Q_t = Q_{\infty} (1 - e^{-rt})$$

where Q_t is the quantity of radioactivity expressed as counts per second in the thyroid at any time, Q_{∞} the asymptotic (*time* = infinity) value of the function Q_t , and r a rate constant. Similarly the descending portion of the observations made over the thigh can be fitted with an exponential function of the form

$$Q_{\text{thigh}} = Q_o = Q_{o0} (e^{-rt})$$

where Q_o is the quantity of radioactivity in counts per second at time t and Q_{o0} is the value of the function corresponding to $t=0$. The parameters Q_{∞} and r for the thyroid curve were evaluated by the method previously described, consisting of plotting $\log (Q_{\infty} - Q_t)$ against t for various estimated values of Q_{∞} until the relationship fits a straight line, and from the slope of this line evaluating r . In the case of the curve for the observations of the thigh, the parameters Q_{o0} and r are evaluated from a straight line fitted to the plot of $\log Q_o$ against t .

Table I shows the values for the rate constant r as evaluated in the same patients from (1) the curves of accumulation of radioiodine in the thy-

TABLE I
Values for the rate constant r determined from urine, thyroid and thigh

Condition	Cases	r (per cent per hour), mean*			Variability of r †	
		Urine	Thyroid	Thigh	(S.D.) ² variance	P
Euthyroid	14	13.8 ± 0.9	13.5 ± 0.9	14.4 ± 1.0	3.18	>0.05
Hyperthyroid	15	37.9 ± 3.3	40.3 ± 3.7	36.2 ± 3.1	26.73	>0.05
Hypothyroid	6	5.93 ± 0.3	6.10 ± 0.5	6.65 ± 0.6	0.70	>0.05

* The value after the \pm is the standard error of the mean.

† The variability of r for each case was calculated as the variance (squared S.D.) of the three estimates derived (urine, thigh, thyroid); the value in the table is the mean variance for the group. The P value was calculated by the method of analysis of variance (10) to determine whether the differences among the determined r 's were significant statistically. A value of $P < 0.05$ is generally considered a good criterion of significance. (For none of the groups was it indicative that the r 's determined in the different ways were different.)

TABLE II

Values for rate constant r determined from urine, blood, thyroid and thigh

Condition	Cases	r (per cent per hour), mean*				Variability of r †	
		Urine	Blood	Thyroid	Thigh	(S.D.) ² variance	P
Euthyroid	6	14.5±1.1	15.0±0.8	12.9±1.1	14.0±1.0	3.76	>0.05
Hyperthyroid	7	39.0±5.3	39.3±5.5	40.6±6.6	37.0±5.1	26.84	>0.05
Hypothyroid	4	6.0±0.5	8.5±1.3	6.0±0.7	6.9±0.5	2.68	>0.05

* The value after the \pm is the standard error of the mean.

† The variability of r for each case was calculated as the variance (squared S.D.) of the four estimates derived (urine, blood, thigh, thyroid); the value in the table is the mean variance for the group. The P value was calculated by the method of analysis of variance (10) to determine whether the differences among the determined r 's were significant statistically. A value of $P < 0.05$ is generally considered a good criterion of significance.

roid, (2) observations over the thigh, and (3) analysis of urinary excretion. Table II shows a similar comparison in those cases included in the first table in which, in addition to the foregoing, the concentration of radioiodine in blood also was determined. It appears from the analysis in both tables that the rates obtained by these various methods are not significantly different from one another either in the individual cases or when the means for the various groups are compared. The actual values obtained in an individual case do show some random variation from one method to another which can be ascribed to the errors in the analytical methods employed and in addition to inaccuracies arising from the methods of curve fitting employed for the estimation of the rates. The rate as determined from analysis of the thyroid curve is the same as that obtained from observations over the thigh, on the urine and on the blood. It is not a measure of thyroidal accumulation rate, but like the rate constant derived from urine represents the proportional rate of disappearance of radioiodine from blood. This follows from the same analysis on which the previous calculations of Keating and associates were based. The rate of disappearance of radioiodine from blood, however estimated, usually varies according to the state of thyroidal activity. No matter how measured it is not a quantitative or direct measure of accumulation of iodine in the thyroid gland, since it must measure the rate

at which iodine disappears from the blood into all sites of disposal including the urine and other tissues as well as the thyroid.

SUMMARY

A method is described for making relative *in vivo* measurements of the quantity of radioiodine accumulated by the thyroid. Accumulation of radioiodine by the thyroid has been measured in 29 individuals having various states of thyroidal function. These measurements have been compared with observations over the extremities (thighs), with measurements of urinary excretion and with analysis of blood for radioiodine.

In vivo observations over the thyroid show substantially more radioiodine accumulated by the thyroids of hyperthyroid individuals than by the thyroids of euthyroid individuals. Euthyroid patients with nodular goiters may at times collect more radioiodine in their thyroids than other euthyroid subjects. Hypothyroid patients show little or no accumulation of iodine in their thyroids.

The accumulation of radioiodine by the thyroid was observed to follow an exponential curve similar in form to the curve of urinary excretion of radioiodine. Observations of radioactivity over the thigh, on the other hand, resembled closely curves constructed from analyses of radioiodine in serum.

Estimation of the exponential rate constant of the curve of radioiodine accumulation by the thyroid was found to have the same value as the rate constant estimated from observations over the thigh, from observations of urinary excretion or from analyses of blood for radioiodine. This rate is regarded as representing the rate of disappearance of radioiodine from blood no matter which method is used to obtain it.

The rate of disappearance of radioiodine from blood varies according to the state of thyroidal activity. Even when obtained directly from observations on the thyroid *in vivo*, however, it does not provide a direct or quantitative measure of the accumulation of radioiodine by the thyroid, since it must measure the rate of disappearance of radioiodine from the blood into all sites of disposal, including the urine and other tissues as well as the thyroid.

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THE MEASUREMENT OF THE IODINE-ACCUMULATING FUNCTION OF THE HUMAN THYROID GLAND

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At least two distinct mechanisms appear to be responsible for accumulation of iodine by the thyroid gland. The first, called the "iodide trap" by Vanderlaan and Vanderlaan (1), involves the concentration, presumably within the thyroid cells, of inorganic iodide obtained from the circulating blood. The second, called hormonal synthesis, involves the utilization of the trapped iodide for the synthesis of organic iodine compounds (chiefly diiodotyrosine and thyroxine) which are then stored in the colloid in thyroglobulin.

Vanderlaan and Vanderlaan (1), and Taurog, Chaikoff and Feller (2) have shown in the rat that, even after hormonal synthesis has been blocked by an antithyroid drug such as propylthiouracil, the thyroid concentrates relatively large quantities of radioiodine. The radioiodine so concentrated remains in the form of iodide and Taurog and associates have shown that it leaves the thyroid at a rate which parallels the decrease of radioiodine in the blood, suggesting that an equilibrium exists between the two. This ability to concentrate radioiodine can be promptly abolished by the administration of thiocyanate, which thus appears to act as a specific poison to the iodide trap.

The iodide-concentrating function has been studied in the human thyroid by Stanley and Astwood (3), who gave tracer doses of I^{131} some hours after administration of a dose of mercaptoimidazol or propylthiouracil. Significant concentration of radioiodine occurred in the thyroid, reaching a peak in one to three hours, thereafter decreasing and largely disappearing within 24 hours. Administration of potassium thiocyanate after the curve of iodine concentration in the thyroid had reached a maximum was followed

by abrupt disappearance of the radioiodine from the thyroid within an hour.

The foregoing is in marked contrast to the manner of accumulation of radioiodine by the untreated thyroid gland (4, 5). The accumulation of radioiodine in the unblocked thyroid can be expressed by an exponential curve which levels off between 24 and 48 hours and thereafter the quantity present in the thyroid diminishes gradually over a period of several weeks. Hyperfunctioning glands accumulate radioiodine both more rapidly and in larger quantities than normal glands and subsequently lose the radioiodine more rapidly than normal glands. Analyses of thyroid tissue removed 48 or more hours after administration of a dose of radioiodine reveal that more than 90 per cent of the radioiodine present is organically combined (6). Taurog and Chaikoff (7) have shown that the untreated thyroid gland of the rat converts the radioiodine which it accumulates to organic form with astonishing rapidity, 95 per cent of the quantity present in the thyroid being organically bound as early as 15 minutes after the dose has been given. The accumulation curve of the untreated thyroid gland may thus be regarded as reflecting both the iodide-trapping and the synthetic function, but it seems probable that both the maximal point in the curve and the rate at which accumulation occurs reflect hormonal synthesis more than trapping.

The accumulation of iodine by the human thyroid as disclosed by radioiodine has been shown to vary significantly in varying states of thyroid activity (4, 8, 9) and has attracted attention both as an aid in diagnosis of thyroid disease and more importantly as a new approach to the clinical study of normal and disordered thyroid function. It represents a distinctly different aspect of thy-

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roid function than other measures of thyroidal activity ordinarily employed clinically, such as protein-bound iodine, basal metabolic rate and blood cholesterol.

In vivo measurement of the quantity of radioiodine in the thyroid involves a number of technical difficulties. In order to convert the counts recorded by a suitable Geiger counter to absolute terms such as microcuries or percentage of the dose given, *in vivo* measurements must be compared with suitable standards. Account must be taken of the stability and efficiency of the counting tube employed, the geometric relation of the apparatus to the subject, the depth and dimensions of the thyroid, the radioactivity in other parts of the body and such factors as absorption and scattering of photons by the thyroid and the tissues which surround it. *In vivo* measurement is thus subject to observational errors of considerable magnitude.

A number of investigators have dealt with these problems. In 1940 Hamilton and Soley (4) made the first attempt to determine the absolute amount of I^{131} collected in the thyroid. Their method consisted in measuring the radioactivity over the anterior and posterior (seventh cervical vertebra) aspects of the neck. The ratio of these values represented the proportion of radiation from the thyroid which penetrated to the back of the neck. The "correct" value for the radioactivity in the thyroid was calculated from this by comparison with a suitable standard of radioiodine. In 1946 Seidlin, Marinelli and Oshry (10) shielded their Geiger counter with 1.91 cm. of lead and measured the radioactivity through a window in the lead shield. The difference between the reading with the window open and that with the window plugged with a 1.91 cm. thickness of lead represents the radioactivity of the region being measured. The absolute value was then determined by comparing this reading with that of a known amount of I^{131} . In 1947 uimby and McCune (11) made absolute measurements by placing their Geiger counter directly over the neck and comparing these readings with those of known samples in a "phantom."

The iodine-accumulating function of the thyroid has been estimated from *in vivo* observations in various ways: 1) by measuring the quantity of radioiodine maximally accumulated (4, 8, 9);

2) by measuring the quantity accumulated in some selected period (10, 11); 3) by observing the relative form and magnitude of the iodine-accumulation curve (4, 5); 4) by estimating from this curve an arbitrary accumulation gradient (12); or 5) by determining the rate constant of the exponential accumulation curve (5). Since Hamilton and Soley had shown that most of a dose of radioiodine was either collected by the thyroid or excreted in the urine, some observers (13) have employed measurements of the quantity excreted in the urine in a selected period, usually 48 hours, to estimate the proportion of the dose collected by the thyroid. There appears to be need for data regarding the relationships of such diverse observations to one another and their individual validity as quantitative measures of iodine-accumulating function.

Keating, Power, Berkson and Haines (14) estimated from the curve of urinary excretion of radioiodine two values: (a) the asymptotic quantity in the urine, which they termed the "renal fraction"; (b) a rate constant, which they considered to represent the disappearance rate of radioiodine from blood. The product of these values, which they called "renal excretion rate," they regarded as the most direct measure of renal function with regard to iodide excretion. The difference between excretion rate and disappearance rate represented disposal of iodine by all tissues other than the kidneys, and was called "collection rate"; a more exact term would have been "extrarenal disposal rate." In so far as the thyroid is the most important site for the extrarenal disposal of iodide, the latter value should provide an index of the rate of iodine accumulation by the thyroid. In support of the interpretation of Keating and co-workers, McConahey and associates (15) determined the rate of disappearance of radioiodine from blood directly from measurements of samples of plasma and found good agreement with estimates of this value obtained from curves of urinary excretion by the method employed by Keating, Power, Berkson and Haines. Luellen and co-workers (5) observed that the rate constants estimated from curves simultaneously obtained from: 1) *in vivo* observation of radioiodine in the thyroid in counts per second; 2) measurement of radioactivity in peripheral tissues (the thigh); and 3) urinary

excretion of radioiodine, all appeared to be the same as the rate of disappearance of radioiodine from blood as determined directly. It was suggested therefore that *in vivo* observations of radioiodine in the thyroid might be analyzed in the same manner as the data for urinary excretion of radioiodine. Thus the product of the asymptotic quantity in the thyroid (expressed as a fraction of the dose given) by the disappearance rate of radioiodine from blood gives the quantity iodine-accumulation rate analogous to the quantity renal excretion rate.

It is the purpose of this communication: 1) to describe an empirical method for measuring *in vivo* the quantity of radioiodine in microcuries collected by the human thyroid gland; 2) to present methods for estimating the iodine-accumulation rates from such data; and 3) to compare iodine-accumulation rate with other measures of the iodine-accumulating function of the human thyroid which may be derived from *in vivo* observations and studies of urinary excretion after oral administration of radioiodine.

METHODS

Radioiodine emits both beta and gamma radiation. The beta radiation will not penetrate much more than 2 or 3 mm. of tissue before being totally absorbed, whereas a large proportion of the gamma radiation will penetrate the tissue and continue in air for several meters. Since the gamma radiation will be emitted uniformly in all directions, its intensity at any distance from the gland should be inversely proportional to the square of the distance, provided one assumes the thyroid to be small enough to be considered a point source. Hence, by placing the Geiger counter at two different distances, P and Q , from the neck one should obtain two intensities, I_p and I_q , which would be inversely proportional to the squares of the distances, A and B , from the focus of radiation to the effective portion of the counter. However, the measurable distances, P and Q , are only from the surface of the skin to the outer surface of the counter. There remains a nonmeasurable portion, x , which represents the depth of the thyroid from the surface of the skin plus the depth of the effective portion of the counter from its surface. It is obvious then that:

$$A = P + x, \quad 1$$

$$B = Q + x, \quad 2$$

where A and B are the true distances. The relation of these quantities to each other is given by the equation

$$\frac{A^2}{B^2} = \frac{(P + x)^2}{(Q + x)^2} = \frac{I_q}{I_p} \quad 3$$

and x , and hence A and B can be calculated from it when P , Q , I_p , and I_q have been measured.

To quantitate the measurements in millicuries or microcuries, the foregoing procedure is first used with a known standard of I^{131} . The Geiger counter being thus calibrated, it is possible to compute the amount of I^{131} in the thyroid in millicuries or microcuries. It must be added that the background radiation is measured after each setting with a lead cylinder 5 cm. long and 9 cm. in diameter (which absorbs more than 99 per cent of the radiation going from the thyroid to the counter) interposed between the area that is being measured and the counter. This reading includes the cosmic radiation, radiation from the thyroid which has been scattered into the counter from other parts of the body, and direct radiation from I^{131} located in parts of the body other than the area being measured. The measurements were carried out with the apparatus and general procedure previously described by Luellen and associates (5). An empirical correction was made for radioiodine in the neck and not in the thyroid by subtracting twice the value of radioactivity recorded in the thigh. Except in the case of comparatively small accumulation in the thyroid, the latter correction is relatively insignificant.

To determine the extent to which geometric relations, absorption and backscatter affect the measurements, samples of I^{131} solution of similar activity but of different diameters were embedded in paraffin phantoms (1, 3 and 6 cm. diameter and 0.5 cm. deep). It was found that when distances (P and Q) of 25 and 45 cm. were used the "inverse-square" relationship was valid to within ± 5 per cent. When the I^{131} was not more than 3.5 cm. below the surface, absorption of the radiation by the paraffin above the solution was compensated for by the backscatter from below and lateral to the source. For depths greater than 3.5 cm. the absorption was greater than the backscatter and the apparent amount of radioactivity decreased approximately 4 per cent per centimeter of paraffin. Hence it would appear that this method of determining the amount of I^{131} in the thyroid should give results which are correct to within ± 15 per cent.

Serial *in vivo* observations were made of 30 subjects including 11 patients who had myxedema,³ five euthyroid patients who had low-grade thyroid tumors, seven patients who had adenomatous goiter without hyperthyroidism and seven patients who had exophthalmic goiter. One or more *in vivo* observations were made on 26 addi-

³ The 11 cases of myxedema included in this study were all instances of severe spontaneous or postoperative myxedema in which *in vivo* studies did not show any measurable accumulation of radioiodine in the thyroid region. Certain cases of myxedematous states have been observed in which varying degrees of iodine accumulation, sometimes of surprising extent, were observed in the thyroid. For the most part these included cases of thyroiditis, struma lymphomatosa and cyanate goiter. Such cases have been omitted from this study and will be made the subject of subsequent reports.

tional persons: 11 euthyroid patients who had thyroid tumors, six patients who had adenomatous goiter without hyperthyroidism and nine patients who had exophthalmic goiter. In all instances, the urine was carefully collected and analyzed for radioiodine in the manner described by Keating and associates (14).

As in previous studies, both tracer and therapeutic doses are included in the series, careful comparison of the behavior of tracer versus therapeutic amounts in patients receiving both having failed to disclose consistent or significant differences either in the quantity of iodine initially accumulated by the thyroid or in the kinetic relationships involved during the first day or two after administration of the dose.

In the interest of clarity the various values employed in this paper may be defined as follows: From the curve of urinary excretion of radioiodine are derived (1) the renal fraction (Q_{uf}), defined as the asymptotic ($t = \text{infinity}$) quantity of radioiodine excreted in the urine expressed as a fraction of the dose; (2) the disappearance rate (r), defined as the proportional rate at which radioiodine disappears from the blood into all sites of disposal; (3) the renal excretion rate, defined as the proportional rate of excretion of radioiodine by the kidneys (This is obtained by taking the product of the renal fraction and the disappearance rate [$Q_{uf} \times r$]); (4) the extrarenal disposal rate, which in previous publications was called "collection rate," defined as the proportional rate of disappearance of radioiodine into all sites other than the urine. This is obtained by subtracting the renal excretion rate from the disappearance rate ($r [1 - Q_{uf}]$).

From the curve of accumulation of the radioiodine by the thyroid as measured *in vivo* can be obtained (5) the thyroid fraction (Q_{if}) defined as the asymptotic ($t = \text{infinity}$) quantity of radioiodine accumulated by the thyroid expressed as a fraction of the dose; (6) the disap-

pearance rate (r) defined as in the previous paragraph; (7) the iodine-accumulation rate, defined as the proportional rate of accumulation of iodine by the thyroid (This is obtained by taking the product of the thyroid fraction and the disappearance rate [$Q_{if} \times r$]); (8) the extra-thyroidal disposal rate, defined as the proportional rate of disappearance of radioiodine into all sites other than the thyroid. This is obtained by subtracting the accumulation rate from the disappearance rate ($r [1 - Q_{if}]$).

The renal fraction and the disappearance rate were estimated from observations on urine as previously described (14). The thyroid fraction and the disappearance rate have been estimated from *in vivo* observations of the thyroid as described by Luellen and his associates (5), except that the quantities in the thyroid have been expressed in actual terms as percentage of dose instead of in relative terms as counts per second.

Reference will also be made to (9) accumulation gradient, a term coined by Stanley and Astwood (12) and defined by them as the slope of a line obtained by plotting thyroid accumulation of radioiodine in counts per second per 100 microcuries of dose against the square root of time. The value is obtained by dividing the increase in counts per second during a given period against the number resulting from subtracting the square root of time in minutes at the beginning of the period from the square root of time in minutes at the end of the period.

RESULTS

In Table I are summarized the means of the various values estimated from the curve of urinary excretion and from the curve of *in vivo* observations obtained simultaneously over the thyroid. For comparison are included (10) the mean quantities of iodine observed in the thyroid 12

TABLE I
Comparison of various values derived from serial observations of urinary excretion and thyroidal accumulation of radioiodine

Diagnosis	Cases	Basal metabolic rate	Values from urinary excretion curve				Values obtained from thyroidal curve					(10) Quantity in thyroid after 12 hours
			(1)* Renal fraction (Q_{uf})	(2) Disappearance rate (r)	(3) Renal excretion rate ($Q_{uf} \times r$)	(4) Extra-renal disposal rate ($r [1 - Q_{uf}]$)	(5) Thyroid fraction (Q_{if})	(6) Disappearance rate (r)	(7) Accumulation rate ($Q_{if} \times r$)	(8) Extra-thyroidal disposal rate ($r [1 - Q_{if}]$)	(9) Accumulation gradient	
		per cent	per cent of dose	per cent/hour	per cent/hour	per cent/hour	per cent of dose	per cent/hour	per cent/hour	per cent/hour		per cent of dose
† thyroid patients who had thyroid tumors Adenomatous goiter without hyperthyroidism Exophthalmic goiter	11	-26.7 ± 3.2†	86.4 ± 2.0	5.6 ± 0.4	4.7 ± 0.5	0.9 ± 0.3	0					0
	5	0 ± 4.4	68.4 ± 1.2	11.5 ± 1.7	7.8 ± 1.0	3.7 ± 0.7	18.5 ± 4.6	12.5 ± 0.8	2.4 ± 0.7	10.1 ± 1.1	3.1 ± 0.5	14.8 ± 4.2
	7	- 2.9 ± 2.3	57.3 ± 6.3	14.0 ± 1.0	7.8 ± 0.8	6.2 ± 1.3	29.5 ± 4.7	14.2 ± 1.7	4.6 ± 1.3	9.6 ± 0.5	6.0 ± 1.5	23.6 ± 4.2
	7	+32.7 ± 2.6	31.2 ± 5.6	29.7 ± 6.5	8.0 ± 1.0	21.6 ± 6.3	56.3 ± 5.8	34.1 ± 6.7	20.2 ± 5.6	13.9 ± 2.6	24.9 ± 5.1	49.8 ± 5.0

* The numbers refer to the definitions given in text above.

† The cases of myxedema selected all failed to show measurable collection in thyroid. See Footnote 3.

‡ The values given are the means and the standard errors of the means.

hours after administration of the dose, and the accumulation gradient as defined by Stanley and Astwood (12).

Of the ten values shown in Table I, eight vary significantly with variations in thyroidal activity and two do not. Renal excretion rate (3) is regarded as a measure of renal function with respect to iodine. Extrathyroidal rate of disposal (8) measures the rate of disposal of iodine by all tissues other than thyroid, including kidneys. As noted elsewhere, the mean disappearance rates (2 and 6) obtained by the two types of observations do not differ significantly.

Attention is called particularly to the magnitude by which the mean values differ in various states of thyroid activity. The renal fraction (1) is twice as high in the euthyroid patients as in the group having exophthalmic goiter but only a fourth higher in myxedema than in the euthyroid patients. The thyroid fraction (5) on the other hand is 3.0 times as great in exophthalmic goiter as in the euthyroid group. It will be noted that the quantity of iodine actually observed in the thyroid after an arbitrarily selected interval (12 hours), also varies by about the same amount, being 3.4 times as great in exophthalmic goiter as in the euthyroid group.

Disappearance rate (2 and 6) is 2.6 times as high in exophthalmic goiter as in the euthyroid group, extrarenal disposal rate (4) is 5.5 times as high in exophthalmic goiter as in the euthyroid group, while the accumulation rate (7) differs by a factor of 8.4 and Stanley and Astwood's accumulation gradient (9) by a factor of 8.1.

It will be noted that the values for the group having adenomatous goiter without hyperthyroidism are to some degree intermediate between euthyroid and hyperthyroid groups. It was observed that large adenomatous goiters tended to show increased avidity for iodine even in the absence of clinical evidences of hyperthyroidism.

The foregoing data were accumulated from serial *in vivo* observations obtained over a period of several days. Such serial observations provide a method of evaluating disappearance rate as described by Luellen and associates (5) and also for assigning a value to the thyroid fraction. Sufficient observation to enable one to construct a reasonably accurate curve is quite time-consuming both to patient and to observer and ties up

the apparatus for the greater part of two days. Even so, there is likely to be an annoying hiatus in the curve unless observations are also made during the night. For these reasons a less time-consuming method for evaluating iodine-accumulation rate might have some advantages.

Since the rate of disappearance of radioiodine from blood can be calculated from measurements over thyroid, peripheral tissues, urine or blood, one should be able to calculate with reasonable accuracy the value of thyroid accumulation rate from (a) a single accurate measurement over the thyroid made during the period of radioiodine accumulation, and (b) the value for disappearance rate calculated from urine as described elsewhere. The thyroid fraction can be approximated from the curve of urinary excretion and a single *in vivo* measurement from the equation $Q_t f / Q_u f = Q_t / Q_u$ where Q_t is the quantity measured in the thyroid, Q_u the quantity excreted in the urine up to the time of the thyroid measurement, $Q_t f$ the thyroid fraction and $Q_u f$ the renal fraction. Accumulation rate estimated in this way is probably less accurate than an estimate based on serial observations *in vivo*, for the reason that a value based on a series of such measurements is less dependent on the error of any one observation than is a value based on a single observation.

In Table II are shown mean values for accumulation rate for the same groups of cases in-

TABLE II
Comparison of accumulation rates estimated
by two methods

Group	Cases	Accumulation rate		Means of individual differences
		Estimated from serial <i>in vivo</i> observations over the thyroid	Estimated from urinary curve and one <i>in vivo</i> observation over the thyroid	
		per cent/hour	per cent/hour	per cent/hour
Euthyroid patients who had thyroid tumors	5	$2.4 \pm 0.7^*$	2.6 ± 0.8	$+0.2 \pm 0.2$
Adenomatous goiter without hyperthyroidism	7	4.6 ± 1.3	4.2 ± 0.9	-0.4 ± 0.5
Exophthalmic goiter	7	20.2 ± 5.6	18.0 ± 4.4	-2.2 ± 1.6

* The values given are the means and the standard errors of the means.

TABLE III

Accumulation rates estimated in a second series of patients from urinary excretion and single observations in vivo over the thyroid

Group	Cases	Basal metabolic rate	(1) Renal fraction	(2) Disappearance rate	(4) Extrarenal disposal rate	(7) Accumulation rate
		per cent	per cent of dose	per cent/hour	per cent/hour	per cent/hour
Euthyroid patients who had thyroid tumors	11	- 4.0±3.2*	64.9±0.8	9.6±0.3	3.4±0.2	2.6±0.3
Adenomatous goiter without hyperthyroidism	6	+ 0.8±2.1	50.9±8.2	11.8±2.1	6.2±2.2	4.7±2.0
Exophthalmic goiter	9	+32.9±3.4	27.8±6.1	28.6±5.3	22.8±6.3	19.8±5.5

* The values given are the means and the standard errors of the means.

cluded in Table I, comparing estimations based entirely on *in vivo* measurements with estimations in the same cases made in the manner just enumerated. The values obtained with each method agree fairly well with each other.

Table III shows accumulation rates for a second group of cases, estimated from urinary excretion curves and single *in vivo* observations over the thyroid. For comparison the various values obtained from the urine alone are also included. The same relationships noted in the first group (Table I) hold here; namely, that the most striking differences are encountered in the accumulation rate and the extrarenal disposal rate.

If all of the iodine given to the patient either appeared in the urine or was collected by the thyroid, then (a) renal fraction (1) plus thyroid fraction (5) should equal 100 per cent; (b) extrarenal disposal rate (4) should equal accumulation rate (7); and (c) extrathyroidal disposal rate (8) should equal renal excretion rate (3). However, it is found that this is not the case. Renal plus thyroid fractions consistently total less than 100 per cent, extrarenal disposal rates are consistently larger than accumulation rates, and extrathyroidal disposal rates are consistently larger than renal excretion rates. The difference between the sum of renal and thyroid fractions and 100 per cent will provide some measure of the fraction of the dose disposed of elsewhere than in kidneys or thyroid. The rate of disposal by tissues other than kidneys or thyroid can be approximated by extrarenal disposal rate (4) minus accumulation rate (7) or alternatively by extra-

thyroidal disposal rate (8) minus excretion rate (3). The "tissue rate" so estimated by either method for any case would have the same value providing the estimate of disappearance rate obtained from thyroid is identical with that obtained from urine, a circumstance which is unlikely owing to the errors in the observations.

In Table IV are given the means of the foregoing values for those cases in which serial observations over the thyroid were made. It is of interest that the fraction of the dose unaccounted for is about the same (12.5 per cent to 13.6 per cent) in all four groups of cases and that the mean rate of extrarenal disposal for the group having myxedema is of about the same order of magnitude as the estimates of "tissue rates" for the other groups. These values would represent any radioiodine excreted in feces, sweat or expired air as well as any radioiodine fixed in tissues such as muscle or liver. These values will also, unfortunately, bring into prominence any loss of iodine in the collection of urine, any error resulting from the methods of measurement employed and any error inherent in the methods by which the various values were derived. Estimates of disposal of iodine by tissues other than kidneys and thyroid vary considerably from case to case, and for the reasons given they must be regarded as relatively inaccurate and probably too high. On this account it is not apparent from this small

TABLE IV

Disposal of iodine by tissues other than kidneys and thyroid

Group	Cases	Fraction of dose unaccounted for	Extrarenal disposal rate (4) - accumulation rate (7)	Extrathyroidal disposal rate (8) - renal excretion rate (3)
		per cent of dose	per cent/hour	per cent/hour
Myxedema*	11	13.6±2.0†	0.9±0.3	
Euthyroid patients who had thyroid tumors	5	13.1±2.9	1.5±0.4	2.3±1.1
Adenomatous goiter without hyperthyroidism	7	13.2±3.5	1.6±0.8	1.9±1.2
Exophthalmic goiter	7	12.5±4.3	1.4±1.1	5.9±2.1

* In the group having myxedema the values given are (a) 100 per cent—renal fraction and (b) extrarenal disposal rate alone, there being no thyroidal accumulation.

† The values given are the means and the standard errors of the means.

series whether any significance should be attached to the relatively greater value for tissue rates in cases of exophthalmic goiter.

COMMENT

Previous studies have dealt with various aspects of the kinetic behavior of radioiodine, on the basis of observations of urinary excretion of radioiodine (14), the concentration of radioiodine in the blood (15) and the quantity of radioiodine in the thyroid or peripheral tissues as measured *in vivo* in counts per second (5). The present study adds to these some *in vivo* observations which are expressed in absolute terms. The results enumerated permit a comparison of various measures in a search for the most appropriate and precise method of evaluating quantitatively the capacity of the thyroid to accumulate iodide by means of observations made with radioiodide. An ideal measure of this function of the thyroid should be expressed in terms which are independent of the dose of radioiodine given and should reflect the accumulation of iodide by the thyroid alone, uninfluenced by irrelevant changes which might occur in the kidneys or in other tissues, or in water exchange.

Measurement of the quantity of radioiodine in thyroid or urine. Direct measurement *in vivo* of the quantity of radioiodine in the thyroid after some interval of time might at first glance appear the most appropriate measure of iodine-accumulating function. This is not true for the reason that the thyroid must in effect compete with the kidneys and other tissues for the limited quantity of iodine provided in the tracer dose. The quantity of radioiodine accumulated by the thyroid in any given period, as well as the quantity eventually or asymptotically accumulated, is a relative quantity, depending on the function of the kidneys and other tissues as well as on the iodine-accumulating function of the thyroid itself. On this basis even if the thyroid function with respect to iodine accumulation remains quite constant, a variation in function of the kidneys or perhaps of other iodine-collecting tissue should alter the picture of iodine accumulation by the thyroid. Thus, if the ability of the kidneys to excrete iodine was decreased, the amount of iodine accumulated in any given time by the thyroid should increase and so should the total amount eventually accu-

mulated. These relationships may be important even if pathologic alteration of the function of the kidneys or other tissues with respect to iodine is not involved. In euthyroid persons, the kidneys and other tissues account for the disposal of three-fourths or more of a tracer dose of radioiodine and quite physiologic fluctuations in the function of the kidneys or other tissues might modify significantly the quantity of radioiodine accumulated by the thyroid.

These considerations may account for the fact that, as judged by the comparative magnitude of the variations encountered in various states of thyroidal activity, neither the quantity of radioiodine in the thyroid at a selected time (12 hours) nor the asymptotic quantity in the thyroid (the thyroid fraction [5]) appears as significant as several other measures of iodine-accumulating function listed in Tables I and III. Measurements of the quantity of radioiodine in the urine, probably because they reflect the quantity in the thyroid indirectly, appear less significant.

Measurements of rates. Disappearance rate (2) reflects accumulation of radioiodine by the thyroid only indirectly, since it measures the disappearance of radioiodine as iodide from the blood into all tissues into which it goes, including kidneys and other tissues as well as thyroid. Disappearance rate varies from one group to another (Table I) by about the same degree as the quantity in the thyroid after 12 hours or the thyroid fraction (5).

Extrarenal disposal rate (4) is one step closer to a direct measure of iodine-accumulating function. It measures the disposal of radioiodine by all tissues other than the kidneys. Inasmuch as the thyroid is the most important of these, except in hypothyroid or athyroid persons, it appears to be a fairly reliable index of iodine-accumulation rate, subject only to inaccuracies resulting from variations in the removal of iodine by tissues other than the kidneys. It is perhaps somewhat paradoxical but nevertheless it appears to be true that extrarenal disposal rate, which is estimated from urine, appears a more valid measure of iodine-accumulating function in cases of normal or increased thyroid activity than direct estimation of the quantity of radioiodine in the thyroid. This is not the case when thyroid activity is reduced or absent.

The accumulation rate (7), estimated directly from serial observations *in vivo* or approximated from the curve of urinary excretion and a single observation *in vivo* during the period of iodine accumulation, appears to measure iodine-accumulating function *directly*, uninfluenced by variations in the function of the kidneys or of other tissues. Together with accumulation gradient it appears the most significant of the various measures enumerated in Tables I and III.

Accumulation gradient. The value which Stanley and Astwood (12) called "accumulation gradient" does not correspond either to disappearance rate or to accumulation rate as we have defined them, although it most nearly approximates the latter. The numerical value given to accumulation gradient is an arbitrary number without quantitative relation to the physiologic processes involved. Since it is a value obtained by dividing counts per second by a function of time, the numerical value of the accumulation gradient will vary with differences in the sensitivity of the counting apparatus, differences in the quantity of radioactivity given in the tracer and differences in the geometric relationship of counter to thyroid. These factors can all theoretically at least be corrected for, but it is likely that they will make it difficult to obtain figures in different laboratories which can be compared with one another. The mean value of accumulation gradient in the euthyroid cases shown in Table I is only about a third as large as Stanley and Astwood's figures, despite the fact that the values were corrected for both quantity of radioactivity and geometric relationship. This lack of agreement probably reflects differences in the sensitivity of the apparatus, and perhaps also a difference in the value of the microcurie employed as a standard.

The accumulation gradient has the great advantage that it can be obtained without continuing observations long enough to evaluate the asymptotic value of the accumulation curve. This advantage was employed by Stanley and Astwood to evaluate the antithyroid potency of various drugs under conditions which would have precluded direct estimations of iodine-accumulation rate, since the accumulation curve was modified by the addition of the drug a few hours after the experiment had been begun.

Significance of accumulation rate. The radioactivity contained in the tracer given to a patient initially may be regarded as labeling only the quantity of iodide given in the dose. Once sufficient time has elapsed for absorption to have occurred and for the dose of labeled iodide to reach equilibrium in the blood and the body fluids, the radioactivity in the tracer may be considered to label all of the iodine in the blood and body fluids which is in the same chemical form as the dose given (that is, as iodide). On this basis accumulation rate measures the proportion of all of the iodide in blood and body fluids which is being accumulated in the thyroid per hour. The iodine-accumulation rate of the thyroid is thus expressed in terms which are independent of the tracer dose given as well as independent of variations in the removal of iodine by other tissues, such as the kidneys.

Accumulation rate has a connotation somewhat similar to "clearance" as used in studies of renal function. It could be used interchangeably with clearance providing the volume of distribution of iodine in blood and in the fluids of the body in equilibrium with the blood did not vary significantly. Accumulation rate relates the iodine-accumulating function to the total stock of available iodide in the body as a whole. It is more reasonable to suppose, however, that this function is more directly related to the *concentration* of iodide in the blood circulating through the thyroid than to the total *quantity* of iodide in the blood and in distant parts of the organism which may be in equilibrium with the blood. Furthermore, it is likely that the volume of distribution of iodide may vary in different persons and in various pathologic conditions. For these reasons, *thyroidal iodide clearance*, expressed as the volume of plasma cleared of its iodide content per minute, may prove to be a more accurate measure of iodine-accumulating function than accumulation rate.

Accumulation rate is estimated from observations on the thyroid alone, whereas clearance of iodide by the thyroid must be derived from simultaneous observations on both thyroid and blood. Thyroidal iodide clearance can be obtained by multiplying accumulation rate by the volume of distribution of iodide, if the latter is known. In the absence of a measure of volume of distribution,

IODINE-ACCUMULATING FUNCTION OF THE THYROID

TABLE V
Comparison of accumulation rate, thyroidal clearance of iodide and volume of distribution of iodide

Case	Diagnosis	Basal metabolic rate	Iodine accumulation rate	Thyroid iodine clearance	Volume distribution of iodine	
		per cent	per cent/hour	cc./min.	total liters	cc./kg.
1	Adenomatous goiter without hyperthyroidism	-14	3.6	13.5	22.8	330
2	Adenomatous goiter without hyperthyroidism	-5	1.0	3.0	18.0	240
3	Adenomatous goiter without hyperthyroidism	+5	0.6	2.2	22.0	414
4	Exophthalmic goiter	+28	23.2	68.0	17.6	290
5	Exophthalmic goiter	+42	10.0	20.4	12.2	210
6	Exophthalmic goiter	+34	24.6	69.0	16.9	270
7	Exophthalmic goiter	+29	50.7	228.1	27.0	500
8	Exophthalmic goiter	+21	13.5	28.8	12.8	230

the thyroidal clearance of iodide may be estimated by a simple modification of the standard formula for renal clearance.⁴ In Table V are given values for thyroidal iodide clearance (determined directly) and iodine accumulation rates for eight cases in which both blood and thyroid data were available. Also given are values for the volume of distribution of iodide estimated from clearances and accumulation rates.⁵ It will be observed that

⁴ The standard formula for renal clearance

$$\frac{\text{concentration in urine} \times \text{volume of urine}}{\text{concentration in blood}}$$

becomes $\frac{Q_t}{B}$ where Q_t is the quantity of radioiodine accumulated in the thyroid in an appropriate interval of time and B is the mean concentration of radioiodide in the blood during the same interval. Q_t is obtained by taking the difference between the quantity of radioiodine observed in the thyroid at the start of the interval and the quantity present at the end of the interval. Since the concentration of radioiodide in the blood decreases exponentially with time, the mean concentration of radioiodide in blood for the interval must be evaluated from the formula

$$B = \frac{B_1 - B_2}{\log_e B_1 - \log_e B_2},$$

where B_1 and B_2 are the observed concentrations at the beginning and end of the interval respectively. The clearances in Table V were all based on observations two and six hours after administration of the dose.

⁵ Volume of distribution = $\frac{\text{clearance}}{\text{accumulation rate}} \times \frac{60}{1,000} \times 100$

the clearances and the accumulation rates vary more or less proportionately in the cases studied, and also that the volume of distribution appears to be somewhat larger than the usual value for the volume of extracellular fluid in some cases. The fact that volume of distribution may vary considerably from case to case indicates that accumulation rate may not reflect clearance with complete fidelity and therefore may not be entirely accurate as an absolute measure of iodine-accumulating function. However, accumulation rate may be the most direct and quantitative measure of this function which can be determined from *in vivo* observations alone.

The nature of the iodine-accumulating function. It is important to note that the function of the thyroid appraised directly or indirectly by any of the measures which have been discussed is a very particular one; namely, the accumulation of iodine in the thyroid. Such accumulation probably reflects in large measure the formation of diiodo-tyrosine and thyroxine and their storage in the reservoir of the thyroid follicle.

If one could assume that the total quantity of iodine contained in the thyroid was more or less constant for any short period (that is, did not fluctuate rapidly), then accumulation rate might reflect the rate at which thyroid hormone was being secreted and perhaps might serve as an index of thyroid turnover rate. However, some of the earliest work of Hamilton and Soley suggests that there could be a dangerous fallacy in such assumptions. They observed in two cases of nodular goiters and hypothyroidism that despite clinical evidences which would lead one to infer that the secretion of thyroid hormone by the thyroid was inadequate or nil, the thyroids collected radioiodine in quantities similar to those collected in exophthalmic goiter. We have observed a similar disparity in certain myxedematous states accompanying thyroiditis and cyanotic goiter. Until more is known, therefore, it is probably best to regard iodine accumulation by the thyroid as a function which does not necessarily or invariably parallel the secretion of thyroid hormone.

The iodide-concentrating function measured by Stanley and Astwood (3) in the human thyroid blocked with mercaptoimidazol must be regarded as a thyroidal function involving iodine distinct

from iodine accumulation as the term has been applied here. The iodine concentrated by the blocked thyroid has been shown to be in the inorganic state and probably in some sort of equilibrium with the blood. The rather rapid decrease of thyroidal radioiodine observed in the blocked thyroid takes place at a rate roughly comparable to the disappearance of radioiodine from the blood as we have observed it in cases of myxedema. These considerations lead one to predict that the kinetic relationships shown to exist between radioiodine in urine, blood and thyroid in this and previous papers will not apply in the case of the phenomenon described by Stanley and Astwood.

Finally, it must be emphasized that the methods employed in this paper and the papers which have preceded it for the estimation of rates of disposal of radioiodine in urine, thyroid and other tissues are based on a model which is undoubtedly oversimplified. The values obtained may contain errors arising from factors which complicate the picture, such as the rate of absorption from the gastro-intestinal tract, the time required for equilibrium to become established between the blood and body fluids, and the rate of secretion of radioiodine from the thyroid once it has been accumulated. It is essential, therefore, to regard the values reported as at best first approximations. Nevertheless, the consistency with which independent observations on urine, thyroid, blood and peripheral tissues can be made to match one another in a simple scheme is some justification for assuming that the various interrelationships which have been discussed may be valid ones.

SUMMARY

An empirical method has been described for the measurement in microcuries of radioiodine collected by the human thyroid gland after oral administration.

The iodine-accumulation rate of the thyroid is defined as the proportional rate at which the thyroid accumulates iodine. Two alternative methods have been described for estimating this value. A basis has been given for assuming that this rate applies to all of the iodide in the blood and body fluids in equilibrium with the blood.

Mean iodine-accumulation rate by the thyroid was 2.4 ± 0.7 per cent per hour in five patients

having normal thyroid function and low-grade thyroid tumors, 4.6 ± 1.3 per cent per hour in seven patients having adenomatous goiter without hyperthyroidism and 20.2 ± 5.6 per cent per hour in seven patients having hyperthyroidism.

Accumulation rate has been compared with measurements of the quantity of radioiodine excreted in urine, the quantity collected in the thyroid and the disappearance rate of radioiodine from the blood. Accumulation rate appears to be a more significant measure of iodine-accumulating function of the thyroid than any of these.

The rate of extrarenal disposal of iodide, estimated from the curve of urinary excretion of radioiodine, closely reflects accumulation rate in cases of normal or increased thyroid activity. It appears in such situations to be a valid index of accumulation rate, despite some inaccuracy resulting from variations in the disposal of iodide by tissues other than thyroid or kidneys.

Accumulation rate may be less significant as a measure of accumulating function than thyroidal iodide clearance, determined directly from *in vivo* observations over the thyroid obtained simultaneously with determinations of the concentration of radioiodine in plasma.

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THE VALIDITY OF THE ELECTROKYMOGRAPHIC METHOD FOR MEASUREMENT OF DIAMETER CHANGE OF THE AORTA AND PULMONARY ARTERY DURING CIRCULATORY DISTURBANCE¹

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It has been demonstrated by Henny, Boone, Chamberlain, and their associates that border movements of the heart and great vessels may be recorded with the electrokymograph (1, 2, 4, 7). Confirmation of this work has been reported by others (3, 5, 6). Our studies were designed to investigate the accuracy with which movements of the aorta and pulmonary artery could be recorded with this instrument, and the significance of these movements as measures of dynamic cardiovascular changes during certain acute disturbances of the circulation. For these experiments, acute intrathoracic pressure variation was chosen as the implement for the production of circulatory disturbances. It is a procedure with which a new investigative method may be evaluated by comparison with observations of venous, arterial and pulmonary arterial blood pressure, cardiac output, roentgenokymography, and roentgenographic lung density in control studies or in the published reports of others.

The electrokymograph utilizes the roentgen ray beam of the fluoroscope. When the long axis of a small rectangle of fluoroscopic screen is placed perpendicular to the pulsating border of the heart or a great vessel, the average intensity of illumination of the whole rectangle will vary with the motion of the border. When movements of a great vessel are recorded, the dense shadow moves outward in systole to diminish the intensity and in diastole the reverse occurs. These changes in illumination, when viewed by a sensitive photoelectric tube, are converted to electrical impulses which are recorded by an electrocardiographic apparatus.

¹The Heart Disease Demonstration Section of the U. S. Public Health Service provided the apparatus and part of the funds for this study.

METHODS

Recording methods

The electrokymographic equipment used in this study was of the type described in detail elsewhere (1, 2). An alternating current direct-writing recorder manufactured by the Brush Development Company (8) was used instead of an optically recording string galvanometer. A single stage of amplification was inserted between the photo multiplier tube and the filter.

The response characteristics of the recording apparatus were investigated as a preliminary procedure. To test the frequency response, a circular lead disc was eccentrically mounted on the shaft of a variable speed motor. The edge of the disc was interposed in the X-ray beam perpendicular to and directly in front of the rectangular slot of the phototube. As the disc revolved, a sine wave was recorded.² The recorded wave amplitudes were then measured at varying motor speeds. The results are shown in Table I.

TABLE I

Frequency response of recording instruments, expressed in terms of percentage of amplitude at one cycle per second

Frequency	% amplitude
1	100
2	89
3	79
4	70
5	62
6	55
7	49
8	43
9	38
10	33
15	20
20	13

In a second procedure, the response characteristics of the direct-writing Brush instrument were compared with those of optically recording Cambridge and Sanborn instruments. Using the same eccentric cam, rotated at speeds from 1 to 75 cycles per second, with the original filter and also other filters with which the loss at higher

²The approximation to a pure sine wave is close enough for practical purposes. The mathematical analysis of the wave form generated by the eccentric cam will be presented in a subsequent publication (20).

frequencies was much less than with the original filter, less than 2% variation was recorded at any time between these three instruments, recording simultaneously.

A third procedure was devised to test the relative sensitivity of the various portions of the phototube slot. For this purpose, the phototube output was directly coupled through a potentiometer to a Cambridge string electrocardiograph. A lead sheet was interposed immediately in front of and perpendicular to the phototube slot. Starting with the slot completely exposed, the lead sheet was moved in successive 2 mm. steps (from base

toward free end of tube) until the slot was occluded. The magnitude of string deflection resulting from each 2 mm. movement of the lead sheet was then recorded and measured. The results are shown in Table II.

TABLE II

Relative sensitivity of various portions on the receptor slot of the phototube

Motion in cm.	Recorded amplitude in cm.
0.0-0.2	0.05
0.2-0.4	0.45
0.4-0.6	0.50
0.6-0.8	0.60
0.8-1.0	0.60
1.0-1.2	0.65
1.2-1.4	0.65
1.4-1.6	0.55
1.6-1.8	0.50
1.8-2.0	0.45
2.0-2.2	0.20
2.2-2.4	0.05

From these studies the following conclusions are drawn: (1) If a sine wave of fixed magnitude is impressed upon the phototube, the recorded amplitude is diminished by 50% when the frequency is increased from 1/sec. to 7/sec. and by 75% when the frequency is in-

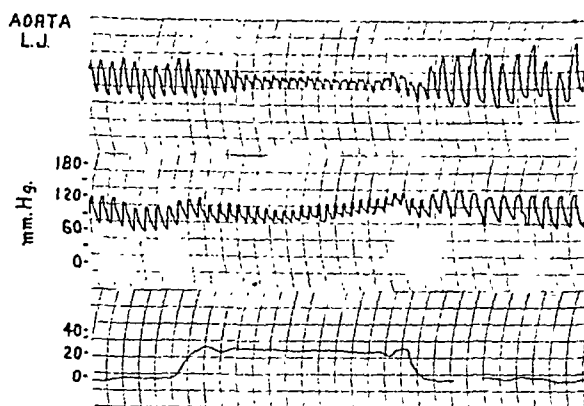


FIG. 1. SIMULTANEOUS RECORDINGS OF AORTIC KNOB PULSATIONS (UPPER), BRACHIAL ARTERIAL PRESSURE (MIDDLE), AND INTRABRONCHIAL PRESSURE. (LOWER) Subject No. 1. Camera speed 1 cm./sec.

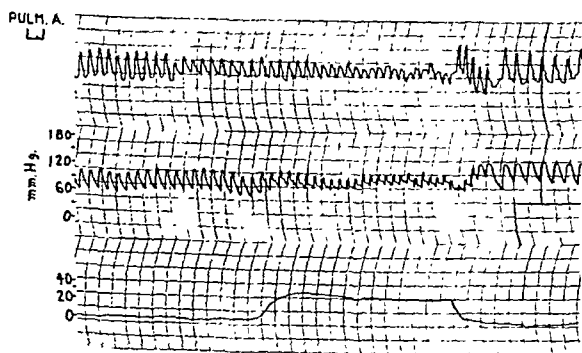


FIG. 2. PULMONARY ARTERY PULSATIONS (ABOVE), WITH BRACHIAL ARTERIAL PRESSURE (MIDDLE) AND INTRABRONCHIAL PRESSURE (LOWER) Subject No. 1.

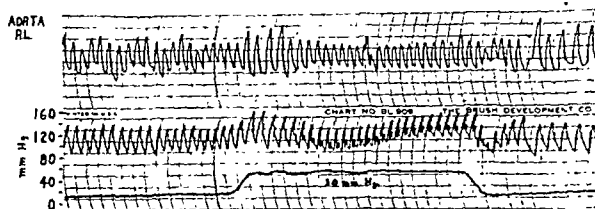


FIG. 3

Same as Figure 1. Subject No. 4. Intrabronchial pressure illustrated is an overlay of the actual recording.

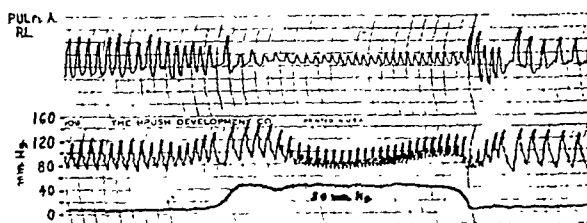


FIG. 4

Same as Figure 2. Subject No. 4.

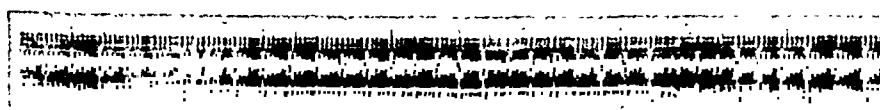


FIG. 5. BALLISTOCARDIOGRAPHIC (VERTICAL TYPE) RECORDING DURING THE STRAINING PROCEDURE Intrabronchial pressure below. Subject No. 10.

creased from 1/sec. to 13/sec. (2) In response to any impulse having a sine wave frequency of from 1/sec. to 75/sec., the records obtained with the direct-writing Brush instrument are identical with those obtained with optically recording instruments. (3) The receptive slot of the photomultiplier tube is 2.4 cm. long. In the zone between 0.6 and 1.6 cm. from the tube-base end, the sensitivity is uniform with a maximum possible error of 15%. In the portion of the slot between 0.2 and 2.0 cm. the maximum possible error is 30%.

Investigative procedure

Ten healthy young adults were selected for this study. None had any symptoms or signs suggestive of heart disease or of pronounced vasomotor instability. The standard straining procedure, a modification of the Valsalva experiment, was performed in the sitting position. Braces were applied to the chest and shoulders to reduce extraneous movement. On command, breathing was arrested in a mid-respiratory position and the recording apparatus was set in motion. After a brief control period, the subject strained against a column of mercury to a pressure of 30 mm. This pressure was maintained for 15 seconds. He then relaxed for ten seconds before resuming respirations. The procedure was then repeated several times. The subject kept his glottis open throughout the experimental procedure.

Employing this standard procedure, electrokymographic tracings were recorded from the pulmonary artery and aortic knob. Intrapulmonic pressure was directly observed by the subject and operator, and recorded by a strain gauge and electrical recorder or capsule and optical recorder. Brachial artery pressure was recorded simultaneously through a Peterson catheter to a capacitance manometer (8, 9). In seven subjects, records were made with the vertical ballistocardiograph during the straining procedure. Representative tracings are shown in Figures 1-5.

In the electrokymographic records, an upward motion parallels outward motion of the great vessels during ejection; the downward curve follows the inward motion of the vessel during diastole. The intrapulmonic, arterial pressure, and ballistocardiographic curves are self explanatory.

RESULTS

The variations of pulse wave amplitude of the aorta and pulmonary artery, as recorded by the electrokymograph during and after the straining procedure, in each of the ten subjects, together with the results of control studies, are indicated in Table III.

Aortic pulsations

The pulse curves recorded from the aortic wall usually remained quite constant during the control period of respiratory arrest. A gradual decrease in amplitude began with the increase in intra-

TABLE III

Subject	Recording	Straining period			Post-straining period		
		4"	8"	12"	1"	4"	8"
No. 1	BP {BP	76	98	106	113	116	100
	PP	81	57	57	67	119	114
	$\Delta p/\Delta d$	1.4	1.7		1.2	0.7	0.7
	EKY {AK	56	33	33	55	166	155
	PA	83	52	50	151	144	120
	Beats/second	1.6	2.0	2.2	1.4	1.1	1.2
No. 2	BP {BP	65	77	62	94	105	98
	PP	93	87	57	50	100	93
	$\Delta p/\Delta d$	1.1	1.1		0.7	0.5	0.5
	EKY {AK	100	81	36	70	215	205
	PA	71	74	37	110	104	115
	Beats/second	2.2	2.9	3.0	3.0	0.8	1.1
No. 3	BP {BP	42	42	36	50	120	91
	PP	52	45	56	47	122	141
	$\Delta p/\Delta d$	1.6	1.6		0.9	0.5	0.6
	EKY {AK	32	27	27	50	243	240
	PA	21	17	21	58	58	154
	Beats/second	2.5	1.2	3.0	3.0	1.3	1.6
No. 4	BP {BP	71	68	92	115	100	89
	PP	147	90	95	90	126	114
	$\Delta p/\Delta d$	0.9	1.2		1.2	1.0	0.9
	EKY {AK	161	78	85	77	134	131
	PA	32	32	40	116	104	80
	Beats/second	1.1	1.4	1.6	1.5	0.8	0.9
No. 5	BP {BP	84	70	69	77	91	85
	PP	100	77	77	70	95	95
	$\Delta p/\Delta d^*$	1.1	3.0		2.8	3.2	1.5
	EKY {AK*	88	25	29	25	30	61
	PA	95	25	18	106	100	155
	Beats/second	1.3	1.3	1.6	2.0	1.0	1.2
No. 6	BP {BP	64	65	73	100	106	100
	PP	80	60	60	60	100	113
	$\Delta p/\Delta d$	1.5	1.9		1.1	0.9	1.1
	EKY {AK	52	32	42	54	116	106
	PA	82	64	75	230	160	136
	Beats/second	1.4	1.7	2.0	1.9	1.1	1.0
No. 7	BP {BP	87	83	87	83	105	111
	PP	100	50	50	50	89	115
	$\Delta p/\Delta d$	0.8	0.9		0.9	0.6	1.0
	EKY {AK	128	56	56	56	143	119
	PA	51	33	29	67	51	51
	Beats/second	2.4	2.4	2.5	2.3	1.6	1.3
No. 8	BP {BP	94	86	78	90	115	115
	PP	100	78	78	61	100	144
	$\Delta p/\Delta d^*$	1.5	1.5		1.0	2.0	2.7
	EKY {AK*	66	53	44	58	49	53
	PA	90	70	50	87	145	117
	Beats/second	1.7	1.9	2.0	2.0	1.2	1.5

* The procedure employed in obtaining these figures was technically faulty (see text).

Ten healthy subjects, aged 20 to 30. Amplitude of electrokymographic recorded pulsations of the aortic knob (AK), pulmonary artery (PA) during and after voluntary straining expressed in terms of percentage of control level. Diastolic pressure (DBP) and pulse pressure, simultaneously recorded from the brachial artery, are also expressed in terms of percentage of control level. Ratio of pulse pressure (Δp) to recorded amplitude of aortic pulsation (Δd) and beats/second are also shown.

TABLE III—Continued

Subject	Recording	Straining period			Post-straining period		
		4"	8"	12"	1"	4"	8"
No. 9	BP {BP	78	67	72	90	96	85
	PP	91	82	78	71	107	107
	$\Delta p/\Delta d$	1.5	1.8		1.0	0.6	0.7
	EKY {AK	61	44	53	74	182	158
	PA	68	84	84	111	121	121
	Beats/second	1.5	1.7	1.9	1.9	1.7	1.4
No. 10	BP {BP	80	68	96	104	105	101
	PP	94	77	58	110	135	135
	$\Delta p/\Delta d$	0.9	1.0		1.6	1.0	1.1
	EKY {AK	100	75	75	69	137	118
	PA	85	90	80	110	95	100
	Beats/second	1.2	1.4	1.6	1.7	1.2	1.2
Median all subjects	BP {BP	78	69	76	92	106	99
	PP	94	77	59	64	104	114
	$\Delta p/\Delta d$	1.25	1.6		1.05	0.8	0.95
	EKY {AK	77	49	43	57	140	125
	PA	76	56	45	110	104	118
	Beats/second	1.7	1.8	2.1	1.9	1.1	1.25

bronchial pressure, and continued until straining ceased. Thereafter, the amplitude increased gradually for the first three or four beats, then rapidly increased to levels higher than in the control period. A pattern of this type was observed in eight of ten subjects. In two subjects (Nos. 5 and 8, see Table III), the amplitude after relaxation did not return to control levels. A technical error, to be discussed below, probably accounted for this.

Arterial pulse pressure

In measurements from the brachial artery, simultaneously recorded, the pulse pressure variations before, during, and after the straining procedure were, in general, parallel to the variations of aortic pulsation amplitude. The characteristic pattern of a fall in pulse pressure during the period of straining, a gradual rise after relaxation, followed by a wide pulse pressure in the later post-straining period, was observed in eight of the ten subjects. In the other two subjects (Nos. 2 and 5) the pattern was atypical only in that the amplitude in the post-straining period reached, but did not exceed, that of the control period.

Diastolic blood pressure

The diastolic blood pressure rose abruptly with the increase in intrabronchial pressure, and fell

when straining ceased. This phenomenon was the result of the transmission to the recording apparatus of pressure derived from the activity of the thoracic and abdominal muscles in raising intrabronchial pressure, and had no direct relation to that component of the recorded pressure derived from the pumping action of the heart. Under these circumstances it is customary to designate the recorded pressure as the *gross* pressure. Subtraction of the intrabronchial pressure from the gross pressure gives the *net* pressure, or that component of the recorded pressure which is dependent upon the activity of the heart. Note that no such correction was necessary for the electrokymographic recordings, since the intrathoracic pressure changes were exerted equally upon both sides of the vessels.

The net diastolic pressure fell rapidly during the first few seconds of straining in all individuals. A considerable rise toward or even above the control level was observed in a few individuals in the late period of straining; in others the fall was sustained. The first beat or two after straining showed little change. A rise above the control level then occurred in eight of the ten subjects.

Pulse rate

From the onset of straining the pulse rate steadily increased. Tachycardia persisted for several beats after relaxation, followed by the abrupt onset of bradycardia.

Pulmonary artery pulsations

Electrokymographic tracings from the pulmonary artery during the period of straining followed the same general pattern as was recorded from the aorta. A reduction in amplitude occurred in all subjects. At the moment of relaxation an important difference was characteristic. A large increase in amplitude regularly appeared immediately upon the return of intrabronchial pressure to the atmospheric level. The later rise above the control level was neither as great nor as consistent as in the aortic recordings. The electrokymographic records from the pulmonary artery resemble the pressure curves recorded by Lauson, Bloomfield and Cournand (10) and by Cournand (11) from the pulmonary artery during a similar straining procedure.

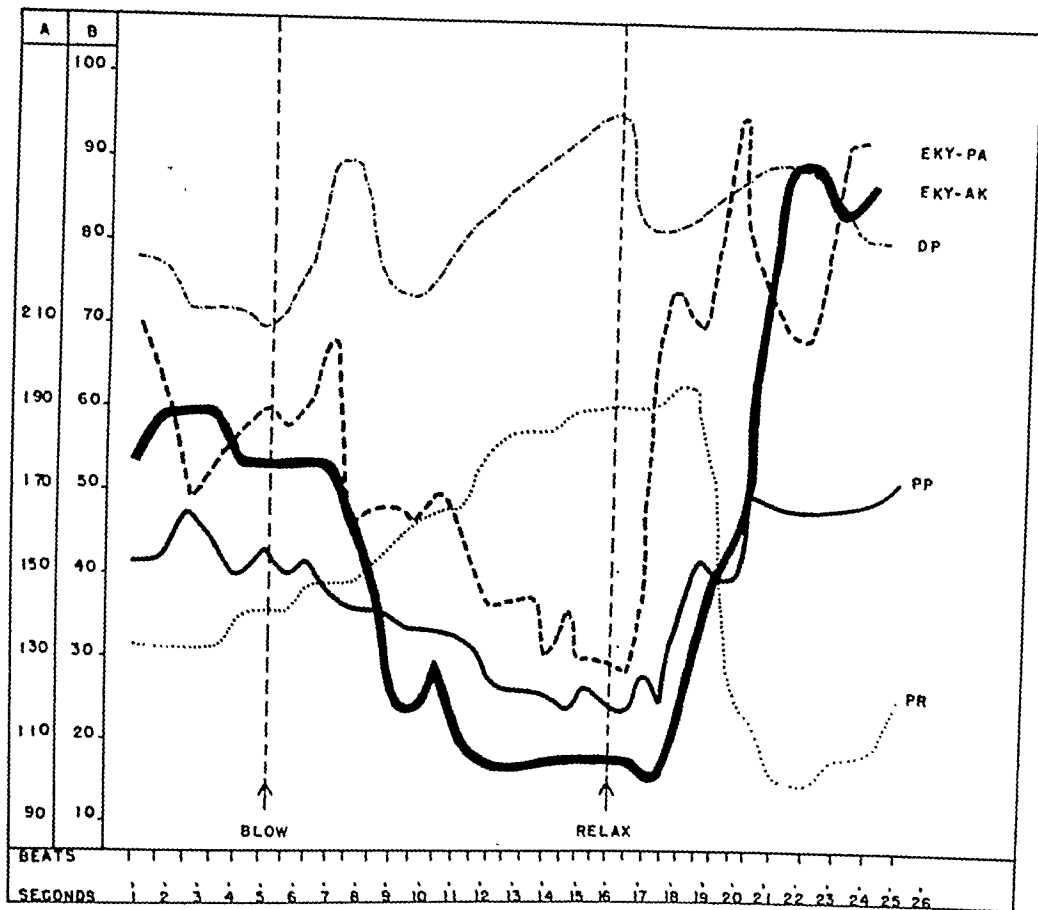


FIG. 6

Subject No. 1. Beat-by-beat amplitude of recorded pulsations of the aortic knob (AK) in mm. (scale B), simultaneously recorded with pulse pressure (PP) and gross diastolic pressure (DBP) in mm. Hg (scale B) and pulse rate (PR) in beats/minute (scale A). Amplitude of pulsations from the pulmonary artery (PA) during a repetition of the straining procedure is shown to illustrate especially the characteristic response in the immediate post-straining period.

Beat-by-beat graphic records from one subject of the magnitude of aortic pulsation with reference to simultaneously recorded pulse pressure, diastolic pressure, and intrathoracic pressure are shown in Figure 6. A similar record of the magnitude of pulmonary artery pulsation of the same individual during a repetition of the straining procedure is included especially to illustrate the major difference between aortic and pulmonary artery response in the immediate post-straining period.

Ballistocardiographic records

Most of the ballistocardiographic records of stroke volume in the sitting position during straining were technically unsatisfactory. The muscular activity necessary to increase voluntarily intrathoracic pressure invariably introduced artefacts into the records. A reduction of complex ampli-

tude was occasionally discernible, followed by large complexes when straining ceased (Figure 5). Application of the Starr formula for determination of stroke volume (12) was rarely possible. Previous ballistocardiographic studies of the stroke volume during straining (13-15) are not pertinent to this problem since the subjects were in the recumbent posture, whereas our subjects were in the sitting posture.

Analysis of results

Stroke change in relative diameter of the aorta and pulmonary artery could be accurately measured by the electrokymographic method if (1) the recorded curves bore a direct, linear relation to the actual movements of the vessel wall, and (2) if the pulsatile change in position of the vessel wall were a true measure of change in vessel diameter. From the data available it is

possible to estimate the degree to which we have approached this ideal.

The relation of recorded curves to vessel wall movements

The response characteristics of the recording apparatus introduced the characteristic imperfections of overdamping. From a study of the frequency response, shown in Table I, it is apparent that this factor must have had an important influence upon recorded wave shape. For example, it was shown that at a frequency of 10/sec., a sine wave of fixed magnitude is recorded only 33% as large as at frequency of 1/sec. If the wave manifested in the dicrotic notch were a sine wave with duration of 0.1 sec., the recorded amplitude would be 33% of that which should have been recorded. Since the wave is actually complex, and usually of shorter duration than 0.1 sec., the magnitude of recorded error is even greater. Other components of the wave pattern are undoubtedly at frequencies of 20/sec. or more. Thus, the finer details of wave shape were smoothed out in the recorded curves and the peaks of the major components were flattened. Some of the differences in wave shape between the recorded aortic curves and the brachial pulse curves (*e.g.*: curves during the late straining period in Figure 1) cannot be explained on the basis of overdamping of the apparatus. Actual differences in wave forms of the two vessels must have been a factor of appreciable importance.

For the comparison of relative amplitudes of recorded waves during the various periods of the straining procedure, overdamping of the degree indicated in Table I should not be expected to introduce a large discrepancy. The duration of the ejection phase of systole in the normal individual with a pulse rate of 1/sec. is 0.25 sec. (16). The curve approximates a half sine wave, therefore the sine wave duration would be 0.5 sec. or a frequency of 2.0/sec. By a similar calculation, the sine wave frequency of systolic ejection at a pulse rate of 2/sec. is 3.0/sec. Although figures are not given, by extrapolation of the curves, the frequency at a pulse rate of 3/sec. is calculated at about 4.0/sec. By calculation from data provided in Table I, it is found that a loss of 19% in recorded amplitude results from

a frequency change of this magnitude. Insofar as the wave patterns of systolic ejection differ in shape from a half sine wave, the loss would be somewhat greater. Since systole ends before the inscription of the last portion of the half sine wave, the loss would be somewhat less. It seems justified to conclude that in these studies, the additional loss in recorded amplitude resulting from the increased pulse rate itself was not greater than 30%, and that amplitude changes of this magnitude, or even of considerably less than this magnitude when pulse rate was taken into consideration, could be considered significant.

In Table II it was shown that the sensitivity of the middle third of the phototube slot was approximately linear. It was usually easy to so place the subject that the shadow of the vessel wall remained in this zone throughout the straining procedure. Hence, non-linearity of the receptor apparatus was not ordinarily an important source of error. Reference was made above to atypical aortic response patterns in subjects Nos. 5 and 8 (see Table III). In reexamining these tracings, it was found that the individual wave patterns were distinctly bizarre in form, with long, flat crests. This pattern was not observed in any other individual studied. It was subsequently demonstrated that similar waves could be produced by allowing the shadow of the pulsating vessel to extend in systole beyond the sensitive portion of the receptor slot. The unusual results in these two individuals are therefore attributed to this technical error. Such faulty technique should be readily avoided and easily recognized when it occurs.

The relation of vessel wall movements to stroke change in diameter

This problem is difficult to subject to objective, quantitative study. Presumptive evidence is offered in several observations. If vessel expansion and contraction were equal in all diameters and the center of the vessel remained fixed, as in Figure 7A, motion of the wall would be in exact proportion to the change in vessel diameter. Even if one side of the vessel were fixed, as in 7B, the same would hold true. If, however, the center of the vessel moved erratically, as in 7C, movement of the vessel wall would be a very inexact measure of the diameter change. Several

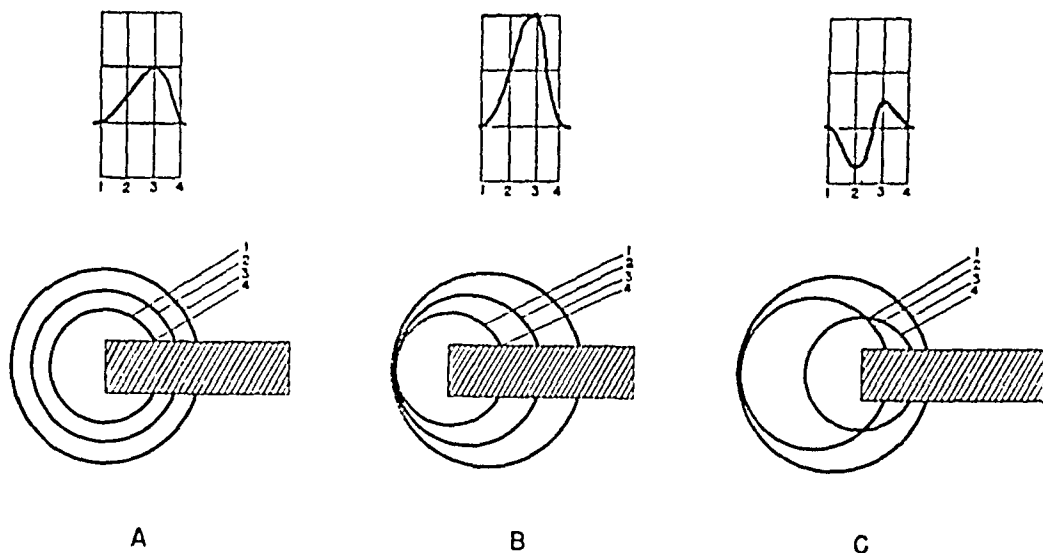


FIG. 7. THE EFFECTS OF LATERAL DISPLACEMENT OF A VESSEL UPON THE CONTOUR OF RECORDED CURVES (UPPER FIGURES)

The cross-hatched rectangle represents the slot of the phototube. Numbers indicate position of the vessel wall at various moments of the pulse cycle.

observations suggest that lateral displacement of the entire vessel was not a major factor in the pulse waves recorded. (a) Respiratory movement undoubtedly causes lateral movement, but this is slow, tending to cause displacement of the entire baseline, which is continuously compensated by the time constant of the apparatus and hence does not greatly influence the amplitude or shape of the individual complexes. In our studies respiration was arrested during the recording period. (b) Rocking motions of the base of the heart might be expected to cause rhythmic lateral displacements of the great vessels. In assessing this factor, a comparison of curves from the left side of the aortic knob with those from the right side of the ascending aorta is of little value, since the differences between the records can evidently be accounted for by the effects of superior venacaval pulsations upon the records from the ascending aorta (6, 7). However, the curves recorded from the aortic knob and from the pulmonary artery invariably show changes in amplitude which correspond qualitatively and to a considerable degree quantitatively with changes in pulse pressure in the respective vessels. If lateral displacement were an important factor, wave motions completely unrelated to pressure changes would be expected. Furthermore, pulsations of large amplitude were regularly recorded from the pulmonary artery in the immediate post-straining period, during which aortic pulsations

were very small. Hence, the motions of the two vessels appear to be independent, each related to the pressure change within the respective vessel, and not a function of a rocking motion of the base of the heart.

In view of these observations, it seems reasonable to conclude that the electrokymographic tracings recorded from the pulmonary artery and aortic knob provide a measure of the relative diameter changes of these vessels resulting from changes in intrinsic vascular pressure. It is obvious that changes in pulse pressure (Δp) should result in changes in aortic cross sectional diameter (Δd). In our studies this was found to be true, but the ratio of pulse pressure to aortic pulsation magnitude ($\Delta p/\Delta d$) varied by as much as 50% in the separate beats of a single individual during a straining procedure (see Table III).

The relation of aortic volume change (Δv) to pressure change (Δp) has been demonstrated by Hallock and Benson (16) in studies of excised human aortas. In individuals of the age group of our subjects (20-30), $\Delta p/\Delta v = k$ where pressures are in the physiologic range. The expression $(\Delta v/\Delta p)V = k$, as employed by Wiggers (17), is essentially the same, since V is a fixed value for each individual tested: namely, the aortic volume at a specified mean pressure.

Katz and his associates (18) found considerable discrepancy between the pressure-volume relationships of the aortas of intact, living dogs

as compared with the same aortas after death. They injected diodrast directly into the aortas and computed aortic volume from the X-ray shadows of films taken at varying levels of blood pressure. Wiggers and Wégria (19) measured changes in aortic diameter directly, with a clamp aortagraph. As in our studies, $\Delta p/\Delta d$ relationships in the separate beats of a single subject showed considerable variation. An inspection of their illustrations shows $\Delta p/\Delta d$ to be 1.0 and 1.4 in beats (y and y of Figure 4, Wiggers and Wégria [19]) of the same approximate pulse pressure and diastolic blood pressures, measured by the technique which we have employed. One would not expect pressure-diameter relationships to exactly parallel pressure-volume relationships. A rough estimate of aortic diameter change to aortic volume change may be deduced as follows:

(1) The relation of aortic diameter change to aortic cross sectional area change approaches linearity. If aortic diameter were 2.0 cm. at a given diastolic pressure, and a unit of systolic pressure increment increased the diameter to 2.1 cm., the resulting increase in aortic area is from 1.0π cm.² to 1.1025π cm.². An area change of ten times this magnitude (1.0π cm.² to 2.025π cm.²) is accomplished with a diameter change to 2.846 cm. Therefore, within the physiologic range specified, diameter change is a linear function of area change within the degree of error in the equation $.1/1 = .846/10$, or about 15%.

(2) Area change would be a linear function of volume change if lengthening were not a factor, but the latter is actually of relatively minor importance. Thus, if in increasing the aortic area from 1.0π cm.² to 2.0π cm.², the aorta were increased in length from 100 cm. to 106 cm., area would be a linear function of volume within the margin of error in the equation $100/1 = 212/2$, or about 6%.

(3) Thus, change in aortic diameter (Δd) appears to be a reasonable approximate linear function of change in aortic volume (Δv). Thus, if $\Delta p/\Delta v = k$, then $\Delta p/\Delta d = k$ should be reasonably accurate.

From the investigations of others, cited above, a roughly linear relationship between change of pressure and change of diameter should have been expected in our subjects. It will be noted that the value of $\Delta p/\Delta d$ is consistently relatively low during the late post-straining period and high during the period of straining. The pulse rate is seen to parallel the values of $\Delta p/\Delta d$. As shown above, the recorded wave amplitude is seen to bear a direct relationship to the pulse rate because of the technical characteristics of the re-

cording apparatus. This technical defect exerts an influence in the direction of the results. The range in results of $\pm 50\%$ in the ratio $\Delta p/\Delta d = k$ is beyond the estimated maximum of 30% error in the response characteristics of the apparatus. A description of certain alterations of the instrument which have resulted in improved linearity of frequency response is now in preparation (20). With such a correction, and with allowances for the differences in wave forms in the aorta as compared with the brachial artery, it seems probable that the variations of $\Delta p/\Delta d$ ratio which we have recorded during acute circulatory disturbance reasonably approximate the actual pressure-volume relationships in the aortas of the individuals studied.

Discussion of the physiologic response to intrathoracic pressure change

The effects of transient increase of intrathoracic pressure, produced by voluntary straining, upon cardiovascular dynamics have been studied intensively by many methods (10, 11, 13, 21-29). The differential pressures in the various heart chambers and great vessels, stroke volume, the distribution of blood mass, and the pulse rate have been shown to be influenced. Interpretation of these data has led to the following conclusions. Increase in intrathoracic pressure is accompanied by a comparable increase in intra-abdominal pressure and results in: (a) An immediate reduction of blood return to the heart from the periphery, with resulting increase in peripheral venous pressure. The blood return from the abdominal viscera is little if at all impeded. (b) The right heart output is consequently reduced with resulting fall in net pressure in the right ventricle and pulmonary artery. (c) The pulmonary blood reservoir is progressively exhausted with reduction in opacity to roentgen rays and subsequent reduction of blood return to the left heart chambers. This is followed by (d) reduced stroke volume of the left ventricle and by reduced pulse pressure and net systolic and diastolic pressure in the systemic circulation with reflex peripheral vasoconstriction and tachycardia. The return of intrathoracic pressure to the atmospheric level is accompanied by a rush of blood into the thoraco-abdominal cavity and right auricle, a sudden marked increase in right heart

output and in pulmonary artery pulse pressure and blood pressure, restoration of the pulmonary blood volume and blood return to the left heart, and restoration of stroke volume, pulse pressure, and systemic blood pressure. In the post-straining period a transient hypertension is normally observed as the result of normal or above-normal cardiac output and persisting peripheral vasoconstriction. A sudden bradycardia regularly occurs as the aortic reflexes respond to this stimulus. All of the cardiovascular functions characteristically return to normal within one or two minutes.

From a careful examination of electrokymographic records alone, most of these physiologic disturbances can be deduced.

SUMMARY

1. Pulsatile movements of the aorta and pulmonary artery were electrokymographically recorded before, during, and after increased intrathoracic pressure produced by voluntary straining. The records obtained were compared with simultaneously recorded brachial artery pressure and with the reported observations of pulmonary artery pressure by others. Ballistocardiography as a control procedure was found to be unsatisfactory.

2. The response characteristics of the electrokymographic apparatus were studied as a preliminary procedure. The sensitivity of the various zones of the receptive slot of the phototube was found to be approximately linear. The frequency response of the apparatus resulted in the characteristic wave shape deformities of an overdamped system. During the various phases of the straining procedure, comparative recorded wave amplitude was sufficiently influenced by this technical factor to necessitate a correction of results for pulse rate.

3. A measure of relative stroke change in diameter of the aorta and pulmonary artery was recorded. Qualitatively these changes were respectively parallel to coincident changes in pulse pressure in the brachial artery in control studies and pulmonary artery in the reported observations of others.

4. The recorded amplitude of aortic pulsations, after correction for pulse rate, bore a quantitative relationship to pulse pressure approximating that

which would be expected between aortic diameter change and pulse pressure.

CONCLUSIONS

From a study of the movements of the aorta and pulmonary artery by the electrokymographic method, most of the cardiovascular disturbances produced by straining may be deduced. Although the measurements are quantitative only in a relative sense, the dynamic changes in the circulation are clearly recorded. This method may provide a useful substitute for direct manometric measurements with catheters in studies in which absolute quantitative measurements of intraaortic and intrapulmonic artery pressures are not necessary. It is a rapid, painless, and easily performed procedure suited to the study of the effects of a cardiovascular stimulus upon each of a large group of subjects. Certain limitations of the value of this method, imposed by the response characteristics of the apparatus in its present form, have been described. From the accuracy with which the nature of the cardiovascular disturbances produced by straining may be deduced from the electrokymographic records alone, the possible application of this method to other studies is implied.

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STUDIES OF THE ROLE OF THE LIVER IN HUMAN CARBOHYDRATE METABOLISM BY THE VENOUS CATHETER TECHNIC. I. NORMAL SUBJECTS UNDER FASTING CONDITIONS AND FOLLOWING THE INJECTION OF GLUCOSE¹

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Previous observations on the role of the liver in carbohydrate metabolism have been made entirely in animals. Recently a method has been developed for obtaining specimens of hepatic venous blood and estimating the hepatic blood flow in human beings (1). This has supplied a tool which makes possible direct investigation of the activity of the liver during various phases of carbohydrate metabolism in man. The present paper deals with the application of this technic to the study of the response of the liver to the fasting state and to the intravenous injection of glucose solution.

METHODS

Hepatic blood flow was estimated by the bromsulfalein method of Bradley (1) in some cases and in others by the urea method of Myers (2). In the few cases where blood flow determinations were not done, it was assumed that the hepatic blood flow was equal to the mean found in our other patients.

The hepatic vein was catheterized by a radiopaque catheter inserted through the right antecubital vein and guided under fluoroscopic observation. The tip was placed centrally in the right lobe in such a position that a free flow of blood could be obtained on aspiration. Occasional checks of position were made during the course of the procedure and at the end of the test period. Bradley (1) has shown that the bromsulfalein extraction may vary from place to place in the same liver. This results in some variation in the estimated hepatic blood flow. In the present study, where serial observations were compared, this type of variation was minimized by keeping the catheter tip in the same location throughout the procedure. At least 5 ml. of blood were withdrawn from the catheter and discarded before samples were withdrawn in order to avoid the possibility of dilution of the sample by saline remaining in the catheter. Peripheral venous and arterial samples were obtained

through indwelling Cournand needles usually placed in the left antecubital vein and the left femoral artery.

Glucose (0.5 gm./kg.) was administered intravenously in 250 ml. of distilled water over a period of 15 minutes.

Blood samples were obtained simultaneously from the artery and hepatic vein before injection and 15, 45, and 60 minutes after the beginning of the infusion. The samples were taken into a sodium fluoride-oxalate mixture and were promptly treated to obtain the necessary filtrates. Glucose determinations were performed by the method of Nelson (3) using a 1:10 zinc hydroxide filtrate. In our laboratory this method has had a standard error of ± 1.0 mg./100 ml. at a level of 100 mg./100 ml. Blood urea was determined on a 10% tungstic acid blood filtrate by the xanthidrol precipitation technic of Engel and Engel (5) and also on a 1:200 dilution of urine. With this method, the standard error of the urea determination was 0.1 mg./100 ml. at a level of 20 mg./100 ml. All determinations were read on a Lumetron photoelectric colorimeter.

CLINICAL MATERIAL

The subjects of these experiments were 15 essentially normal volunteers who were convalescing from acute diseases. All were well nourished and had been on a diet supplying more than 200 gm. of carbohydrate for at least three days prior to the procedure. The subjects were fasted for 18 hours before the tests. In some cases 0.1 or 0.2 gm. of nembutal in 50 ml. of water was given orally about 30 minutes before the procedure. Every attempt was made to keep the subjects comfortable. Venipunctures and arterial punctures were done under procaine local anesthesia. The patients were usually at ease, and most of them dozed during the course of the experiment. As a result of the precautions taken, it seems unlikely that the normal physiological reactions to pain, fear, or anger significantly affected the observations.

CALCULATIONS

The arterio-hepatic venous difference (A-HV) in mg./100 ml. is obtained by subtracting the hepatic venous from the arterial concentration. When the concentration is higher in the hepatic venous blood than in the arterial, the result is a negative quantity, indicating that the liver was losing the substance into the circulation. Where the A-HV value is positive, the liver was retaining the sub-

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stance, either by storing it (*e.g.*, glucose as glycogen) or by metabolizing it.

The hepatic venous system drains not only the liver but also the gastrointestinal tract and the spleen. Strictly speaking, therefore, the value for A-HV represents splanchnic rather than hepatic metabolism. This is discussed below.

An attempt has been made to estimate the amount of glucose formed from protein in the liver, on the basis of the amount of urea released. Using Shaffer's (6) data for the G/N ratios in diabetic dogs, it was assumed that the release of 1 gm. of nitrogen as urea represented the deamination of a sufficient quantity of amino acids to supply 3 gm. of glucose. On this basis 1 gm. of urea is equivalent to 1.4 gm. of glucose. The validity of this calculation is discussed below.

The total splanchnic metabolism is obtained by multiplying the value of A-HV by the estimated hepatic blood flow, expressed as hundreds of milliliters per minute. Myers has shown (7) in man that the injection of glucose solution intravenously at a rate comparable to that used in these observations produces a mean decrease of hepatic blood flow of $10 \pm 3\%$ during the first hour after the infusion. This minor alteration of hepatic blood flow has been disregarded in our calculations.

RESULTS

Hepatic blood flow: Estimations of the hepatic blood flow fell well within the range of normal

as determined by Bradley (1) and by Myers (2) who found mean hepatic blood flows of 865 ± 24 and 800 ± 71 ml./min./M², as compared with 851 ± 71 ml./min./M² in this series (Table I).

Fasting hepatic glucose production: The splanchnic circulation contributed a mean of 3.5 ± 0.7 mg. of glucose per kilogram per minute to the circulation under fasting conditions (Table I).

Fasting hepatic urea production: The mean rate of urea production was found to be 0.21 ± 0.5 mg./kg./min. This may be considered a reflection of the rate of deamination of amino acids and, therefore, it provides a rough estimate of the rate of gluconeogenesis. The urea excreted may be expressed as estimated equivalents of glucose formed. Under fasting conditions the glucose formed from protein represents $7.3 \pm 1.0\%$ of the total hepatic glucose output (Table I). There was no relationship between the glucose output or the blood glucose concentration and the rate of urea production.

The effects of intravenous glucose: After the administration of 0.5 gm. of glucose per kilogram intravenously over a period of 15 minutes, a rise

TABLE I
Estimated hepatic glucose production, urea formation and blood flow under fasting conditions

Subject	Surface area, M ² Wt., kg.	Hepatic blood flow	Glucose output	Urea output	Glucose from protein*	Glucose from protein	Glucose calories	Normal BMR†	Normal BMR from glucose
		ml./min./M ²	mg./kg./min.	mg./kg./min.		%	per M ² /min.	cal./M ² /min.	%
B. B.	1.49 51.7	641†	2.4	.13	.18	7.5	.268	.606	44
E. M. P.	1.54 52.3	711†	4.8	.33	.46	9.6	.648	.625	104
J. W.	1.66 57.0	—	1.6	.09	.13	8.1	.200	.650	31
M. B.	1.58 55.0	—	5.2	.14	.20	3.8	.592	.740	80
L. W.	1.89 67.0	896	6.3	.10	.14	2.2	.916	.649	141
H. M. C.	1.72 62.6	740	1.5	.45	.63	4.2	.220	.656	34
J. W. A.	1.73 67.5	840	1.3	.11	.15	11.5	.204	.655	31
R. C.	1.94 80.5	1210	1.9	.12	.17	9.0	.320	.641	50
W. M.	1.59 50.2	920	6.1	.44	.62	10.1	.760	.650	117
Mean		851	3.5	.21		7.3	.459		70
Standard error		71	0.7	.05		1.0	.094		14.4

* The method and rationale of converting urea excretion into equivalents of glucose produced from protein is discussed in the text.

† BMR estimated from the tables of Aub and Dubois.

‡ Blood flow estimated by the urea method.

TABLE II
Effect of the intravenous injection of glucose on the arterial and hepatic venous glucose levels
(mg./100 ml.)

Subject	Fasting			15 Minutes			45 Minutes			60 Minutes		
	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV
L. L.*	86	111	-25	445	394	+51	185	194	-11	145	175	-30
J. C. B.	85	84	+1	369	341	+28	153	166	-13	74	166	-92
W. P.	74	83	-9	304	271	+33	117	119	-2	83	92	-9
A. P.	89	102	-13	340	317	+23	182	176	+6	144	139	+5
H. McK.	75	90	-15	343	328	+15	190	194	-4	170	151	+19
B. B.	72	82	-10	270	233	+37	181	194	-13	137	137	0
E. M. P.	108	130	-22	331	298	+33	196	193	+3	149	168	-19
M. B.	87	105	-18	270	273	-3†	170	161	+9	133	121	+12
L. W.	84	109	-25	262	264	-2†	149	154	-5	152	148	+4
J. C.	97	112	-15	300	288	+12	234	201	+33	172	187	-15
Mean	86	100	-14	310	290	+20	175	173	+2	135	145	-11
Standard error	3.9	5.4	2.6	12.7	11.4	5.0	11.0	8.8	3.4	11.5	9.3	10.3

* This patient received 100 gm. of glucose, rather than the standard dose of 0.5 gm./kg. The data are therefore excluded from the statistical analysis.

† These samples were taken five minutes after termination of the glucose clysis.

in the arterial glucose level occurred. This gradually fell after the termination of the clysis until it reached normal levels. The shape of the arterial glucose tolerance curve indicated a normal carbohydrate metabolism in all cases (Table II).

The fasting hepatic venous glucose level was higher than the arterial in all cases except one (Table II, J. C. B.). There is no ready explanation for the inconsistency of this single observation.

Fifteen minutes after the clysis was begun, the hepatic venous glucose level was lower than the arterial in all but two cases (Table II, M. B. and L. W.). In these instances, technical difficulties caused a delay in obtaining the samples so that they were not collected until five minutes after termination of the clysis. At this time the arterial glucose level had begun to fall. It seems possible that an outpouring of glucose from the liver, stimulated by the sudden drop of arterial glucose concentration, might account for the negative values of A-HV in these cases.

At 45 minutes after the beginning of the infusion, more than half of the patients had negative value for A-V, and the mean A-HV was not significantly different from zero. At this time the mean arterial glucose level was 175 mg./100 ml. In some subjects, therefore, the liver began to give up glucose at a time when the arterial glucose level was still elevated. Such a

response suggests that regulation of the splanchnic glucose balance may depend partly on the rate of fall of the glucose level in addition to the absolute glucose concentration in the blood.

At 60 minutes the tendency to release glucose from the liver into the peripheral circulation was more marked. At this time, however, the patients were reacting with less uniformity than during the periods immediately after the injection. This

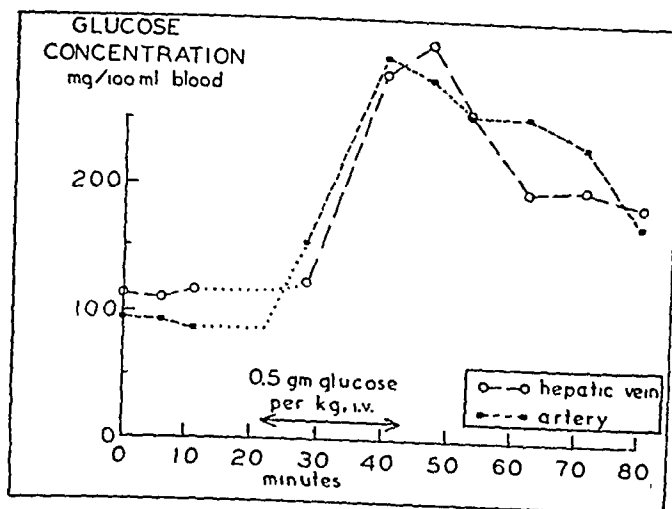


FIG. 1. ARTERIAL AND HEPATIC VENOUS GLUCOSE LEVELS, FASTING AND AT FREQUENT INTERVALS AFTER THE INTRAVENOUS INJECTION OF 0.5 GM. OF GLUCOSE PER KILOGRAM OVER A 15-MINUTE PERIOD

Note the intermittent retention and release of glucose by the splanchnic system during the descending portion of the curve.

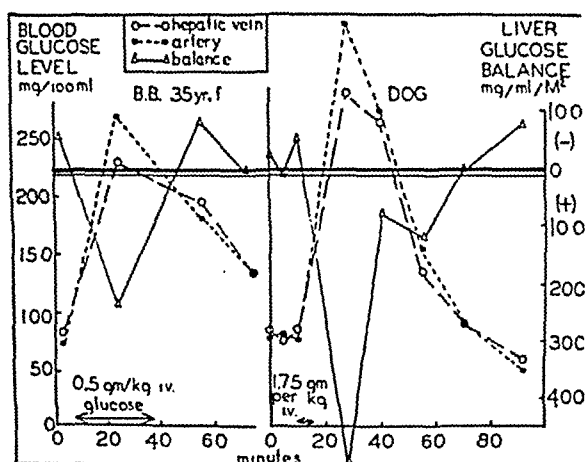


FIG. 2. ARTERIAL AND HEPATIC VENOUS GLUCOSE LEVELS AND ESTIMATED HEPATIC GLUCOSE BALANCE AFTER THE INTRAVENOUS INJECTION OF GLUCOSE IN A HUMAN SUBJECT (LEFT) AND A DOG (RIGHT)

Balance values plotted above the zero line represent retention of glucose, and below the line represent release of glucose by the splanchnic system. The data for the dog are reproduced by permission of Dr. Soskin (8).

is reflected in the relatively large standard error for A-HV.

The observation that the splanchnic system could release glucose even in the face of high blood glucose concentrations suggested that the determining factor might be the rate of fall of the glucose level. To test this, one experiment was done in which samples were collected at frequent intervals. The curves obtained are shown in Figure 1. In this subject the hepatic venous concentrations fluctuated widely around the arterial curve. The significance of these data is discussed below.

The total glucose balance can be calculated by multiplying A-HV by the hepatic blood flow. This has been done for patient B. B. and plotted, together with the arterial and hepatic venous curves, in Figure 2. For comparison an experiment of the same sort in a dog, reported by Soskin *et al.* (8), has been plotted in the same figure. In order to avoid superimposition of the curves, negative balances have been plotted above the double line and positive balances below. The greater excursion of the glucose curve in the dog is probably due to the fact that 1.75 gm. of glucose per kilogram were injected in five minutes, whereas in the patient 0.5 gm. per kilogram was injected in 15 minutes.

The peripheral venous glucose levels were invariably lower than the arterial and were usually lower than the hepatic venous levels under fasting conditions. After the infusion the venous level rose less sharply than did the arterial or hepatic venous concentrations. During the declining phase of the curve, the fall of the venous level was also less rapid. As a result, the concentration of glucose in the peripheral venous blood was usually higher than in the arterial or hepatic venous blood by the end of the observation period. These relationships are shown for a typical case in Figure 3.

DISCUSSION

The hepatic venous system drains not only the liver but also the gastrointestinal tract and the spleen. Strictly speaking, therefore, the value for A-HV represents splanchnic rather than hepatic metabolism. Unfortunately, there is no method available at present for obtaining portal blood in the intact human being. By keeping our subjects basal we have hoped to reduce the metabolism of the extra-hepatic tissues in the splanchnic system to a minimum, and thus achieve a close approximation of the activity of the liver itself. It is possible to estimate the magnitude and direction of the error introduced in the study of hepatic metabolism by our inability to obtain portal ve-

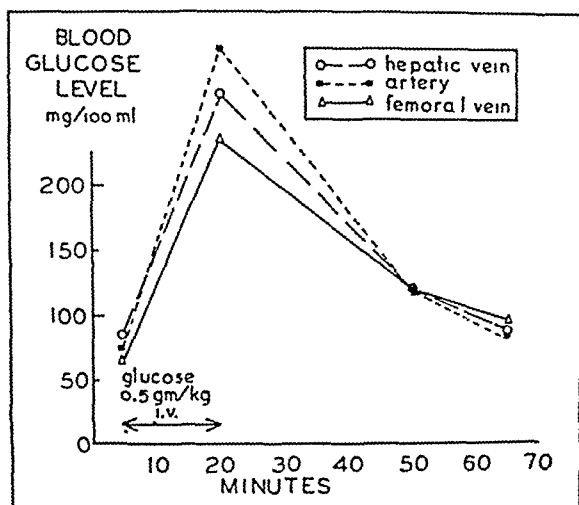


FIG. 3. ARTERIAL, PERIPHERAL VENOUS AND HEPATIC VENOUS GLUCOSE VALUES BEFORE AND AFTER THE ADMINISTRATION OF 0.5 GM. OF GLUCOSE I.V. OVER A PERIOD OF 15 MINUTES

nous blood. Under fasting conditions in man, Sherlock and Walshe (9) and Ravault *et al.* (10) have shown that the portal glucose level is the same as that of the peripheral venous blood. London (11) found a consistently lower concentration of glucose in the portal vein of the dog than in the arterial blood. Since the portal blood contains less glucose than the arterial in the fasting subject, the amount of glucose released by the liver is actually greater than is indicated by the value of A-HV. The data of Soskin *et al.* (8) and of Lipscomb and Crandall (12) may be recalculated to determine the error introduced by using only the arterial and hepatic venous values. Under fasting conditions, the difference between the glucose production estimated from arterial and hepatic venous blood alone and that estimated from arterial, portal and hepatic venous blood, was less than the standard error of the glucose determinations in eight instances. There was a significant difference in two instances. In each case, where a difference occurred the value obtained from A-HV was lower than that from combined portal and arterial blood. After the intravenous administration of glucose to the dog of Soskin *et al.* (8), however, the error rose so that it was significant in every period, averaging 28.9% of the actual value. In every case, the value obtained by the use of A-HV was lower than that obtained using both arterial and portal blood.

In the basal human subject, therefore, it seems probable that values obtained by the methods we have used are quite close to the true hepatic metabolism, although tending to be somewhat lower. After the injection of glucose, it is less accurate to speak of our results as indicating liver metabolism. Even in this case, however, the error is consistent and the major fluctuations introduced are due to the activities of the liver. With these reservations, therefore, we have used the terms "splanchnic" and "hepatic" interchangeably.

The data obtained from normal human subjects agree in all particulars with the observations made on experimental animals. The mean rate of glucose production in the fasting human being (3.5 ± 0.7 mg./kg./min.) falls within the range observed in dogs. Lipscomb and Crandall (12), studying dogs by a method comparable to the

technic we have used, found a mean glucose production of 2.0 ± 0.2 mg./kg./min. In their review of the literature they found reported values obtained by various methods, varying from 1.0 to 4.2 mg./kg./min. in the dog. Bouckaert and deDuve (13) have recently reported a value of $4.0 \pm .75$ mg./kg./min. as the amount of glucose which must be infused to maintain the blood glucose level of a hepatectomized dog. They interpret this figure as equivalent to the amount of glucose released by the normal liver. It is of interest that the mean hepatic blood flow is less in man than in the dog (23 ± 1.5 ml./kg./min. for the human, versus 32 ± 2.2 ml./kg./min. for the dog [12], $p < 0.01$). The fact that the glucose output is equivalent suggests that a larger amount of glucose is released per milliliter of blood traversing the splanchnic circulation in man than in the dog.

Since the rate of release of glucose to the peripheral circulation may be considered as equivalent to the rate of utilization (combustion plus storage as glycogen or fat) of glucose in the peripheral tissues, our patients were utilizing glucose at a mean rate of 3.5 mg./kg./min. This is equivalent to 0.014 cal./kg./min. If calculated on the basis of calories per square meter of body surface, the mean rate of glucose release from the liver accounted for $.459 \pm .094$ cal./M²/min. This represents a mean of $70 \pm 13.9\%$ of the normal basal caloric requirement, calculated from the data of Dubois and Aub, for our patients. Since no direct measurement of the metabolic rate of the subjects was made, it is unwarranted to assume that the glucose output represented 70% of the actual metabolic requirement at the time of the observations. In three cases the rate of glucose production was higher than the total "normal" basal metabolic requirement (Table I), suggesting either that the subjects may not have been basal or that their basal metabolic rate may have been abnormal.

The conversion of urea production into equivalents of glucose production can only be a rough approximation of the true value. It has been estimated that the amount of glucose produced from protein might, under circumstances of maximal conversion, give rise to a G/N ratio as high as 8.0 (4). In order to determine the maximum amount of glucose which could have been re-

leased as a result of the observed rate of urea production, one would have to multiply the figures in Table I by a factor of 2.67. Even under these circumstances, however, the portion of glucose produced by the liver from protein breakdown would be only $19.7 \pm 2.7\%$.

The finding of negative values for A-HV at times when the arterial glucose value was still high is not in agreement with observations by Soskin *et al.* (8) in the dog. These observers found that the hepatic glucose balance did not become negative until the arterial glucose level had fallen to control levels. There are certain differences in the technics used which might account for the lack of agreement. The dogs utilized by Soskin were anesthetized and had been subjected to extensive surgical procedures, whereas the patients we were observing were in an essentially normal state. It is therefore possible that the animals observed by Soskin were not so sensitive to changes in the blood sugar level as were the human subjects. The fluctuations observed in A-HV can be explained by postulating that the hepatic glucose balance is sensitive both to the arterial glucose level and to the rate of fall of the glucose concentration. The influence of the rate of fall of the arterial glucose concentration may be partly mechanical, since a sudden fall of the intravascular concentration of dextrose would cause a diffusion of extracellular glucose back into the sinusoids, thus producing a negative value for A-HV.

It therefore seems probable that the rate of fall of the arterial glucose level plays a part in regulating the release of carbohydrate from the liver. This is well illustrated in the single case in which samples were obtained every ten minutes. Thus the liver, by intermittently releasing glucose to the circulation, brakes the descent of the blood glucose concentration and protects against the development of hypoglycemia.

It was noted that the peripheral venous glucose levels tended to be somewhat lower than those of the corresponding hepatic sample. This indicates that the peripheral tissues remove more glucose per milliliter of blood supplied to them than does the liver. No statement can be made regarding the relative total amounts of glucose involved, however, since no measurements of the

blood flow through peripheral tissues were attempted. After the injection of large amounts of glucose, the rate of fall of the arterial glucose concentration was greater than that of the peripheral venous content, so that toward the end of the period of observation many patients had higher venous than arterial levels. This can probably be explained entirely on the basis of diffusion of glucose from the intercellular tissue spaces back into the peripheral venous system as the arterial glucose level fell below the concentration of the substance in the interstitial fluids.

SUMMARY AND CONCLUSIONS

The hepatic catheterization technic has been used in studying the role of the normal human liver in carbohydrate metabolism both in the basal state and after the administration of glucose intravenously. Under fasting conditions, the splanchnic system releases glucose to the circulation at a mean rate of 3.5 ± 0.7 mg./kg./min. Of this, approximately 7% can be accounted for on the basis of gluconeogenesis from protein. The hepatic glucose production could account for a mean of 70% of the total normal basal metabolic requirement of the patients.

After the administration of glucose by vein, there is an immediate retention of glucose by the liver. Subsequently, as the arterial glucose level falls, glucose is released from the splanchnic circulation. The stimulus for this release appears to be both the arterial glucose level and the rate of fall of the glucose concentration. Evidence was obtained of the release of glucose from the liver at a mean arterial glucose level of 175 mg./100 ml.

It appears that the carbohydrate metabolism of the normal human being is similar to that of experimental animals on which previous studies have been made.

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METABOLIC STUDIES ON PROTEIN-DEPLETED PATIENTS RECEIVING A LARGE PART OF THEIR NITROGEN INTAKE FROM HUMAN SERUM ALBUMIN ADMINISTERED INTRAVENOUSLY^{1,2}

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I. INTRODUCTION

Protein depletion has long been recognized as detrimental to many bodily functions. The problem is particularly urgent in the chronically ill patient who is unable to ingest an adequate caloric or protein intake. During the past decade the search for parenteral substitutes for oral protein has proceeded apace, although frequently without due regard for the fact that retention of nitrogen can be expected only when a certain critical caloric intake supplement has been made available in the form of carbohydrate and fat (1-3).

Mixtures of the essential amino acids, protein hydrolysates, and human plasma have been used with some success (4-6). All, however, possess certain disadvantages. Protein hydrolysates and synthetic amino acid preparations may give rise to vomiting and other reactions; also the rapidity with which they are deaminated and excreted in the urine suggests that they are poor sources of

nitrogen (4, 6). Plasma, especially when derived from large pools, may carry serum jaundice; and its relatively high content of sodium is also a source of difficulty in patients who tend to collect edema. Moreover, the utilization of intravenously administered plasma protein has been the subject of some controversy during the past few years. It is generally conceded that positive nitrogen balances can be established in both animals and man by the intravenous administration of homologous plasma, provided that the dose is in excess of the minimum protein requirements. It is felt by some investigators (7, 8) that the positive balance obtained is an illusory affair and that the actual utilization is poor in some instances. On the other hand, Whipple and co-workers (9) believe that parenteral plasma protein can supply all the requirements for maintenance over a considerable period of time. This implies that there is an exchange between the tissues and the injected protein and that this exchange satisfies the need of endogenous metabolism.

When purified human serum albumin became available in large quantities through the Red Cross program, it was recognized to have certain advantages over other materials in common use. Briefly these are: its low content of sodium, the general freedom from reactions when given intravenously in moderate amounts, and the fact that it can be prepared free from the hazard of viral jaundice. It carries the stigma, however, of being deficient in both tryptophane and isoleucine (10, 11). This deficiency has been partially rectified by the inclusion of acetyltryptophane as a stabilizer. Yet in view of the reported deficiencies, studies on the nutritive value of albumin for man should be pertinent. Eckhardt and others (12) were unable to maintain nitrogen balance when

¹ Part of this work was carried out under a contract between the University of Rochester School of Medicine and Dentistry and the Atomic Energy Commission. Partial support was received from the Committee on Growth, National Research Council, acting for the American Cancer Society, and from the Hochstetter Fund of the University of Rochester School of Medicine and Dentistry.

² The albumin used in these studies was prepared by the American National Red Cross from the blood of voluntary donors. This material has been supplied to investigators by the American Red Cross as part of its National Blood Program. The opinions and conclusions are those of the authors and do not necessarily reflect the policy of the National Blood Program.

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37.5 Gms. of albumin a day, administered orally together with an adequate caloric intake, were the sole source of protein. Supplements of dl tryptophane and dl isoleucine did not improve utilization. However, when 50 Gms. of albumin were given, nitrogen was retained. Two patients were maintained in positive balance on 37.5 Gms. of albumin daily when the albumin was given intravenously. They concluded that albumin could maintain nitrogen equilibrium in man when given either orally or intravenously in sufficient amounts and that supplements of tryptophane or isoleucine were unnecessary. It should be recalled, however, that maintenance of equilibrium, or positive nitrogen balance, in short periods of this sort is quite a different problem from satisfying growth or maintenance requirements over a long period of time.

Albright *et al.* (13) have offered indirect evidence for the utility of injected albumin or plasma protein in protein nutrition. In a study of hypoproteinemic patients they found retention of a sufficient quantity of phosphorus to indicate a conversion of as much as 50 per cent of the injected protein into cellular protein.

In view of the evidence at hand, it seemed pertinent to set up a series of experiments which would put the current theories to further test, particularly with respect to the fate of the albumin in the depleted patient. Subjects were chosen who were in need of protein and who were not acutely ill, since it was thought that the utilization of albumin might be more efficient in such individuals. The amount of albumin administered was placed at a level well above the probable basic protein requirements. In order to assure the best possible opportunity for conversion, the caloric intake was kept at as high a level as the patient would tolerate. None of the diets were protein-free, but, except in one instance, the amount of diet protein was too low to maintain nitrogen balance.

II. PATIENT MATERIAL AND PROCEDURE

G. V. was a 21-year-old white male who had developed rheumatic heart disease with mitral stenosis and insufficiency at the age of five years. Cardiac decompensation had occurred on two occasions, during both of which there had been transient jaundice, perhaps secondary to chronic passive congestion of the liver. At the time of the present studies, the rheumatic disease

did not appear to be active, and the patient was compensated. He was, however, malnourished and appeared chronically ill. The basic diet provided 3300 calories and 2.6 Gms. of nitrogen. This was supplemented by the addition of 60 Gms. of beefsteak protein in two preliminary periods and subsequently by 60 Gms. of human albumin intravenously each day for a ten-day period.

E. G. was a 43-year-old white female who had been ill for ten months before the metabolic studies were begun. Her illness was characterized by migratory polyarthritis, irregular bouts of low-grade fever, transient accumulations of fluid in the pleural cavities, anorexia, and loss of weight. The diagnosis was never fully established in this patient, although it was believed that she had a generalized vascular disease, suggestive of periarteritis nodosa. At the time the metabolic observations were started, she was emaciated but afebrile, and appeared to be in a quiescent phase of her disease. She did not tolerate the type of low protein diet used in the other patients and a liquid formula providing 1200 calories and 8.2 Gms. of nitrogen was substituted in its place. Following a five-day control period, the intake was supplemented for 12 days by 60 Gms. daily of intravenous albumin.

W. R. was a 28-year-old female with a history of acute and chronic alcoholism. She was asymptomatic at the time of study and had no clinical evidence of liver disease. An inadequate diet associated with alcoholism had brought her weight below optimum. The basic diet in her case provided 4.2 Gms. of nitrogen and 2600 calories. Albumin was administered intravenously as in the other patients in the amount of 60 Gms. daily for 15 days.

Eugene G., a 26-year-old white male, presented the syndrome of neurocirculatory asthenia, but had no signs

TABLE I

Sex, height, weight, age and composition of basic diet of subjects receiving intravenous albumin
(All dietary values on per diem basis)

Patient	G. V.	E. G.	W. R.	Eugene G.
Sex	Male	Female	Female	Male
Ht. (cm.)	182.2	156.0	169.0	172.0
Wt. (kg.)	56.1	38.7	57.2	56.6
Age (yr.)	21	43	28	26
Daily calories	3355	1225	2643	2282
Carbohydrates (Gms.)	727	109	487	450
Fat (Gms.)	46	59	68	47
Protein (Gms.)	11.8	55.5	23.5	17.6
Found by analysis				
N Gms.	*2.630	8.160	*4.200	2.600
Ca Gms.	.360	1.540	.421	.278
P Gms.	.914	1.470	1.140	.406
K Gms.	—	—	3.200	3.860
Na Gms.	—	—	—	0.118
Cl Gms.	—	—	—	0.159

* Represent average values based on 5 days of continuous sampling.

of organic disease. There was a history of suboptimal caloric intake for more than a year but no recent loss of weight. He was physically the most normal subject of the group and probably had no significant protein depletion at the time of the experiment. His basal diet provided 2300 calories and 2.6 Gms. of nitrogen per diem.

A summary of the basic data on these patients plus the analytical values found on their control diets is presented in Table I. The albumin used was also analyzed and found to contain in each 100 cc. (a 25 per cent solution) 3.90 Gms. of nitrogen, 14 mg. of phosphorus, 13.84 m.eq. of sodium, and .78 m.eq. of chloride.

III. METHODS

All subjects were admitted to the special Metabolic Ward for study where well-controlled balances could be conducted. Diets were weighed and cooked under the supervision of the dietician, and any food refused by the patients was saved for analysis. The major emphasis was placed upon the determination of the balance of nitrogen, calcium and phosphorus. In addition the potassium balance was determined for W. R. and the sodium, potassium and chloride balances were determined for Eugene G. Urine was collected in periods of 24 hours. Stools were saved in five-day pools and separated by carmine markers. The intake for elements entering into the calculation of the balance was determined either by analysis of sample diets duplicating in all respects the food as served to the patients, or by the method of continuous sampling as described by Bassett and Van Alstine (14). The analytical procedures were carried out as follows: nitrogen in the urine, diet and stool by macro-Kjeldahl; calcium of the diet, urine and stool by the gravimetric procedure (15); daily analyses of the urinary phosphorus were made by the method of Fiske and Subbarow (16); phosphorus in the diet and feces by the gravimetric method (15); sodium and potassium were determined by the internal standard method of flame photometry (17); chloride by the open Carius method (18). Serum proteins were determined colorimetrically (19) and fractionated by Howe's method; they were also measured by a method based on the electrophoretic pattern (20). Urinary protein was determined by a modification of the biuret method (19). The dye T 1824 was used to estimate plasma volume using Gibson's technique (21). Hematocrits and erythrocyte sedimentation rates were done as described by Wintrobe (22).

IV. RESULTS

Nitrogen balance

The three subjects (G. V., W. R., and Eugene G.) who received a basic diet containing 4.0 Gms. or less of nitrogen a day were in negative nitrogen balance during the control periods, although from inspection of Figures 1, 3, 4 it is clear that they tended to approach equilibrium in the final preliminary days. E. G. (see Figure 2) who re-

ceived slightly more than 8 Gms. of food nitrogen of good quality each day, retained nitrogen for the first five days, in spite of the low caloric intake. In her case, the need for protein was obviously great and the reduction in calories apparently had not reached the critical level (1) where retention of nitrogen is impossible. All four patients were found to be in markedly positive nitrogen balance during the first few days of albumin injections and in none was there an appreciable increase in the urinary nitrogen during this time. This is in marked contrast to the prompt increase in urinary nitrogen in G. V. (Figure 1) when he was fed beefsteak protein in an amount equivalent to the albumin injected. After the first few days on albumin, the nitrogen

NITROGEN BALANCE AND URINE PROTEIN DURING INTRAVENOUS ALBUMIN

G.V. AGE 21 MALE UNIT NO. 182390

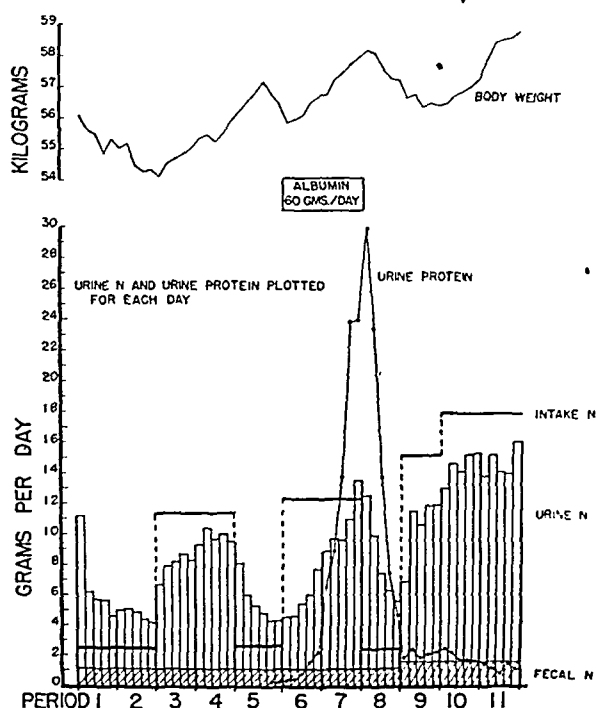


Fig. 1

Positive nitrogen balances are indicated by the clear areas between the intake line and the columns representing the sum of urine and fecal excretion. An extension of these columns above the intake line denotes a negative balance.

The urine protein is given as grams of protein per 24 hours.

NITROGEN BALANCE AND URINE PROTEIN DURING INTRAVENOUS ALBUMIN

E.G. AGE 43 FEMALE UNIT NO 267749

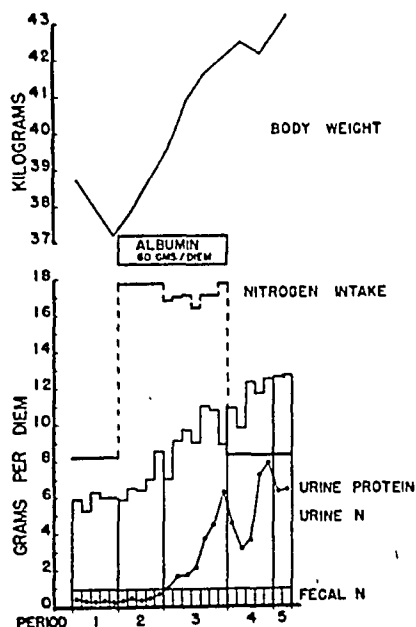


FIG. 2

For legend, see Figure 1.

of the urine began to increase and rose slowly but progressively until the experimental period was terminated. In only one instance, however (the case of G. V.), did the urinary nitrogen become so great as to produce a negative nitrogen balance. The reason for this negative balance is undoubtedly to be found in the marked proteinuria which this man developed. When the injections of albumin were discontinued, the urinary nitrogen did not immediately decline to the level of pre-albumin days, but remained well above that level so that the negative balance of the five post-control days was in excess of that encountered in a preliminary period of equal length. This can hardly be interpreted other than as catabolism of some of the albumin still remaining in the body.

Proteinuria

All the patients excreted protein in the urine at some time during the course of administration of albumin, but in only one subject, G. V., was massive proteinuria encountered (see Figures -4). In his case distinct traces of albumin, although in amounts too minute for precise measurement, had appeared in the urine from time to time prior to parenteral albumin administration.

On the fourth day of the injections, however, there was a sharp rise in urinary protein which by the tenth day reached a maximum of 30 Gms. in 24 hours. This wastage of protein was undoubtedly responsible for the negative nitrogen balance at the end of the experimental period. The electrophoretic pattern of the urinary protein showed chiefly albumin with a trace of alpha globulin. The phenomenon of proteinuria was less marked in the remaining three patients, and its onset tended to be more delayed. At maximum E. G. excreted about 7 Gms. daily, Eugene G. 5 Gms. daily, while the amount excreted by W. R. did not rise above 3 Gms. in 24 hours. On cessation of injections of albumin, the proteinuria in these patients declined sharply or ceased altogether within five or six days.

Body weight

The two male patients (G. V. and Eugene G.) each gained about 2 kg. in weight during the time albumin was given, while the two women showed

NITROGEN BALANCE AND URINE PROTEIN DURING INTRAVENOUS ALBUMIN

W.R. AGE 28 FEMALE UNIT NO. 238476

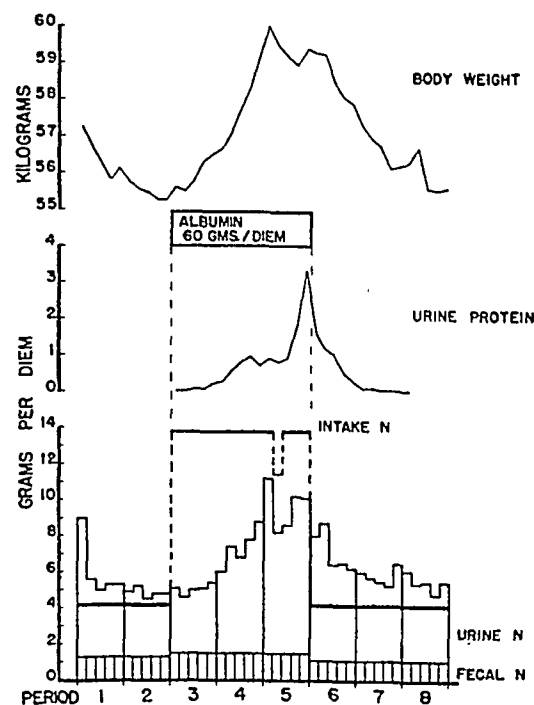


FIG. 3

For legend, see Figure 1.

NITROGEN BALANCE AND URINE PROTEIN DURING
INTRAVENOUS ALBUMIN

EUG. G. AGE 26 MALE UNIT NO 188107

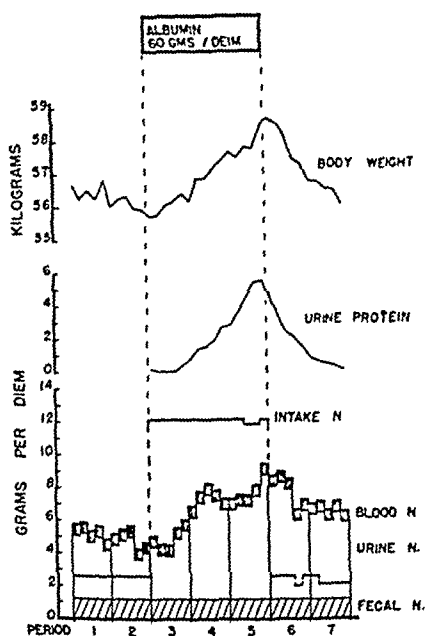


FIG. 4

For legend, see Figure 1. Blocks labeled "blood N" represent nitrogen removed in blood taken for analysis.

gains of about 5 kg. (Figures 1-4). That the major factor in the weight gains of all patients was an expansion of the extracellular fluid volume, is suggested by the fact that the portion of albumin nitrogen apparently retained as protoplasm was quite inadequate to account for the weight gain in any patient. The mechanism of the large gain in extracellular fluid seen in the female patients was probably different in each case. Evidence to be brought out later suggests that albumin leaked into the extracellular fluid of E. G. where it tended to bind water. W. R., on the other hand, showed little evidence for the retention of large amounts of albumin in the extracellular fluid. She did, however, take large amounts of salt and water during this time and sodium retention was felt to be partially responsible for the accumulation of fluid. It will be noted (Figure 3) that some decrease in weight occurred before the injections of albumin were stopped, and that this coincided with a moderate restriction of the intake of salt and water.

Plasma protein and plasma volume

The rise in the concentration of plasma albumin above the control level was of the order of 2 Gms. per cent (Figures 5-8). The maximum concentration of albumin, 6.3 Gms. per cent, occurred in Eugene G. The concentration of globulin decreased as the albumin rose, but this was apparently an effect of dilution, since calculations of the total circulating globulin based on the increase in plasma volume showed no change. An expansion of the plasma volume to about 25 per cent above the initial values occurred in all cases but there was no change in the cell volume (except in the case of Eugene G. where rather large quantities of blood were removed for analysis). As a consequence the hematocrit fell. There was also a decrease in the erythrocyte sedimentation rate, presumably related to the higher concentration of plasma albumin.

EFFECT OF INTRAVENOUS ALBUMIN ON SERUM PROTEINS,
BLOOD VOLUME, HEMATOCRIT AND ERYTHROCYTE
SEDIMENTATION

GV AGE 21 MALE UNIT NO 182390

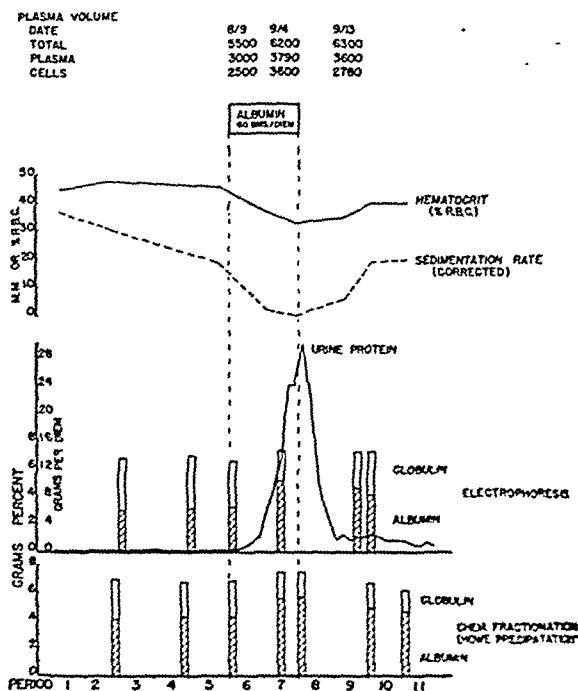


FIG. 5

Clinical course

In the categories already discussed, *i.e.*, nitrogen balance, proteinuria plasma proteins, plasma volume, hematocrit, and sedimentation rate, one notes a striking similarity in the reactions of the four patients. The sharply differing clinical responses, however, demonstrate the effect of the condition of the subject on the fate of the injected albumin.

Patient G. V. tolerated the injections of albumin well until the seventh day when he began to complain of loss of appetite and nausea. On the tenth day of albumin he was observed to be jaundiced. At no time was there any clear evidence of heart failure although he was known to have rheumatic heart disease and had had cardiac decompensation on two previous occasions. The jaundice and anorexia subsided promptly on cessation of the injections, and the remaining period of observation passed without incident. The intense proteinuria exhibited by this man may have acted as a safety valve to prevent expansion of his plasma volume to the point where it would have precipitated cardiac failure.

Patient E. G. ran a stormy clinical course. On the third day of albumin she became febrile and during the last few days of injections, as well as

EFFECT OF INTRAVENOUS ALBUMIN ON SERUM PROTEINS, BLOOD VOLUME, HEMATOCRIT AND ERYTHROCYTE SEDIMENTATION

E.G. AGE 43 FEMALE UNIT NO 267749

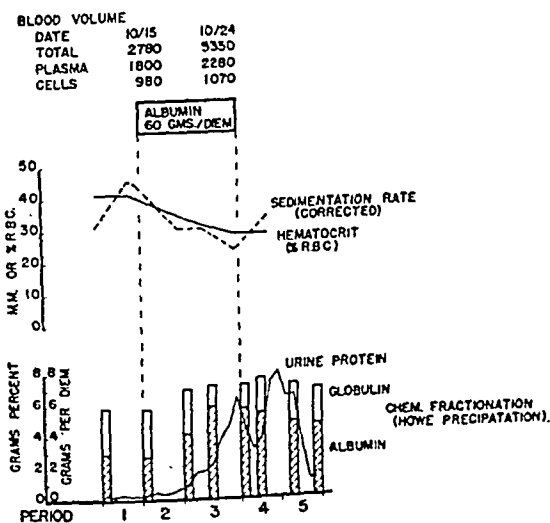


FIG. 6

EFFECT OF INTRAVENOUS ALBUMIN ON SERUM PROTEINS, BLOOD VOLUME, HEMATOCRIT AND ERYTHROCYTE SEDIMENTATION

WR AGE 28 FEMALE UNIT NO 238476

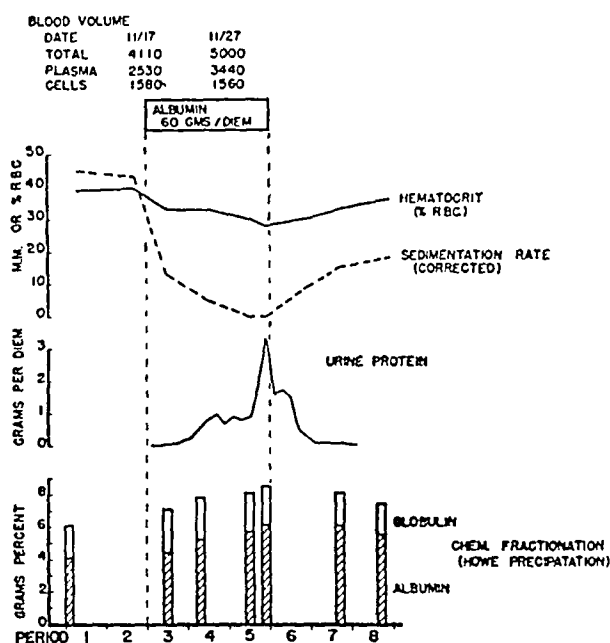


FIG. 7

in the post-control period, there was remittent fever, with spikes as high as 40° C. A left peroneal palsy was observed on the fifth day, and on the sixth day edema became evident, which increased progressively throughout the rest of the experimental period. Small bilateral pleural effusions, which were present on admission, increased in size and a pericardial effusion was noted shortly after the patient returned to the general ward. Both the fever and the peripheral neuritis were felt to be part of her disease process. There seems little doubt, on the other hand, but that the edema, increasing pleural effusions, and the pericardial effusion were secondary to the administration of albumin. Obviously the remarkable gain of 5 kg. in weight in 12 days was due to the increasing anasarca.

Patient W. R. was unaffected by the injections until the eighth day. At this time she complained of a feeling of fullness in the abdomen and loss of appetite. During the evening of the 11th day, she had an attack of nocturnal paroxysmal dyspnea and rales were heard at the bases of both lungs. Restriction of salt was enforced beginning with the 12th day, following which there was a

prompt diuresis and a decrease in weight. Nevertheless, exertional dyspnea persisted and on occasion scattered rales were heard at the base of the right lung. Both symptoms and signs disappeared within two days after stopping the injections of albumin. It should be recalled that this patient had no evidence whatever of organic heart disease, and the syndrome of left ventricular failure appeared to be secondary to the increase in plasma volume, undoubtedly related to the intravenous administration of albumin.

Eugene G. received albumin intravenously for 15 days during periods 3 through 5 in the experiment. In period 4 the patient ran a low grade fever and had a mild diarrhea probably due to an intercurrent infection. During the last six days of the experimental period he too complained of a feeling of fullness in the abdomen, became anorexic, and vomited occasionally. It was difficult to evaluate his symptoms because of his exaggerated emotional responses and it was considered

EFFECT OF INTRAVENOUS ALBUMIN ON SERUM PROTEIN, BLOOD VOLUME, HEMATOCRIT AND ERYTHROCYTE SEDIMENTATION

EUG. G. AGE 26 MALE UNIT NO 188107

PLASMA VOLUME	
DATE	2/19 3/5
TOTAL	3960 4650
PLASMA	2220 3140
CELLS	1740 1510

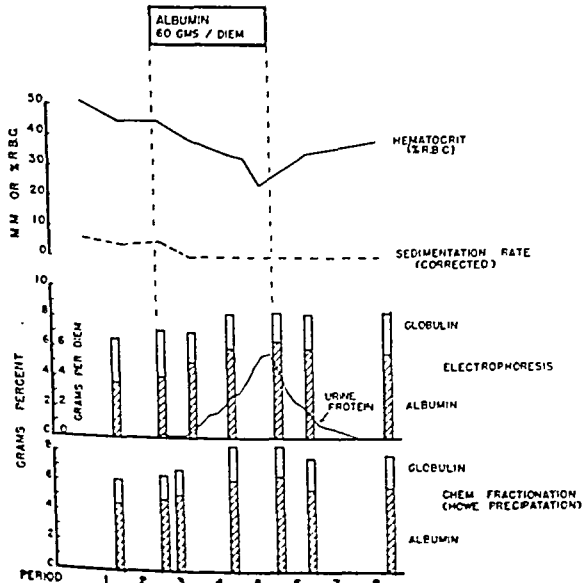


FIG. 8

CALCIUM AND PHOSPHORUS BALANCES

G.V. AGE 21 MALE UNIT NO. 182390

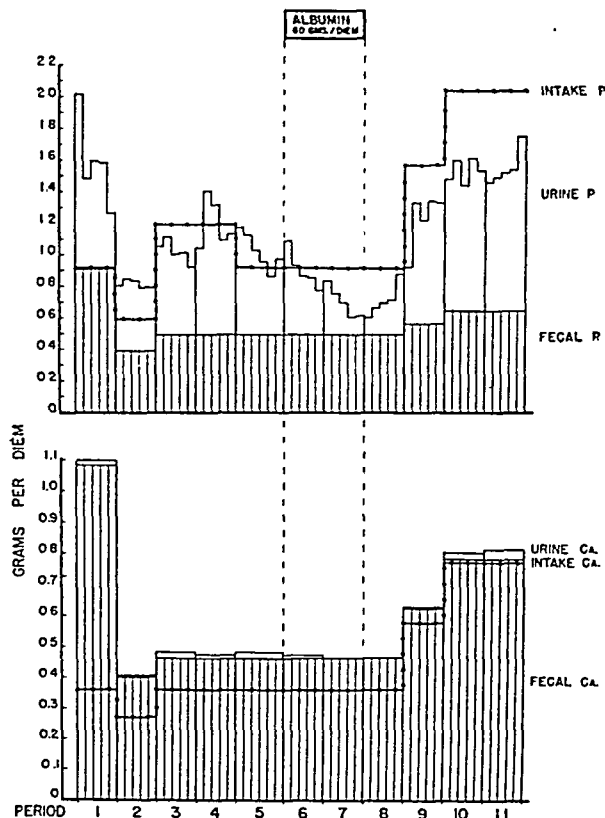


FIG. 9

Positive calcium and phosphorus balances are indicated by clear areas between intake line and the columns representing the sum of urinary and fecal excretions. An extension of these columns above the intake line denotes a negative balance.

improbable that albumin therapy was the chief basis of his discomfort. The objective effects were the gain in weight and the proteinuria which have been considered previously. Neither of these was of a magnitude to cause concern.

Calcium and phosphorus balances

Phosphorus balances were done on all patients primarily to estimate the amount of nitrogen which was released or bound in the catabolism or anabolism of tissue protein. This can be done only when the exchange of phosphorus with calcium is taken into consideration (23) and for this reason calcium balances were also carried out. The essential data are plotted in Figures 9-12.

CALCIUM AND PHOSPHORUS BALANCES

E.G. AGE 43 FEMALE UNIT NO. 267749

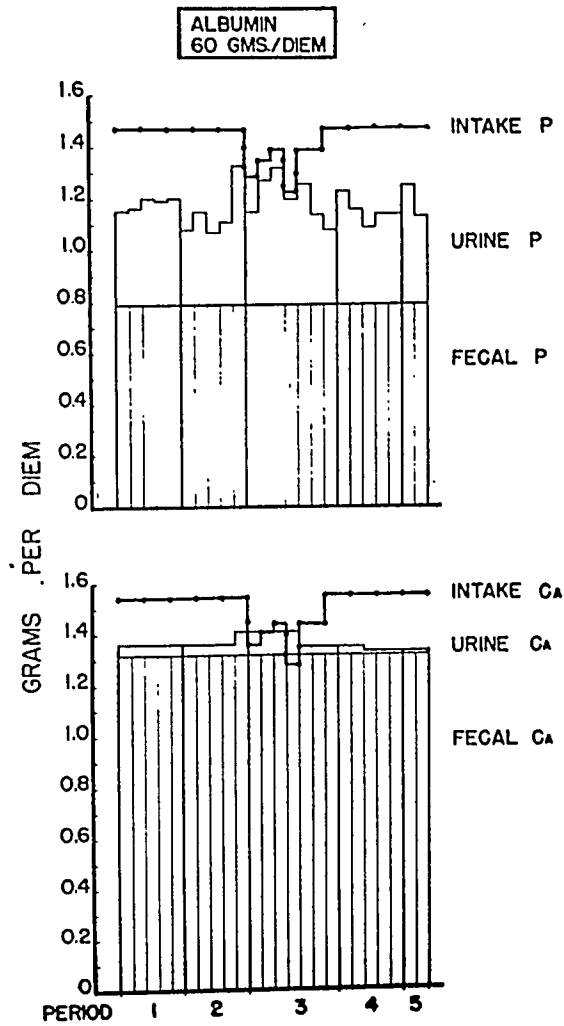


FIG. 10
For legend, see Figure 9.

Because of the dietary and other differences in the programs to which these patients were subjected, it will be necessary to include a brief description of each case.

The rheumatic patient, G. V., received an intake of phosphorus of 0.914 Gm. and calcium of 0.36 Gm. daily during the control periods. The phosphorus intake was increased appreciably when the beef supplement was given, but was not increased materially by albumin which contains very little phosphorus. The phosphorus balance followed the expected course during the control periods and at the time when beefsteak was given (*i.e.*, it correlated well with the nitrogen balance). In periods 6 and 7 when albumin was injected, one notes a gradual decrease in the excretion of uri-

nary phosphorus; and this continued throughout the post-control period (period 8). Because of the low intake of calcium, the balance was negative and the excretion in the urine very low. In periods 7 and 8, evidently as a consequence of the administration of albumin, calcium completely disappeared from the urine and its disappearance coincided with the lowest values of urinary phosphorus. An adequate diet was given in periods 9, 10, and 11 with retention of considerable nitrogen and phosphorus. Calcium was present again in the urine but the balance still remained slightly negative.

The calcium and phosphorus in the diet of E. G. were moderately high, 1.54 and 1.47 Gms. a day, respectively. Some of the irregularity in the intake (Figure 10) is the result of small losses through vomiting. Retention of both elements occurred in the control period. Injection of albumin did not produce much reduction in urinary phosphorus nor change in the phosphorus balance, a fact which constitutes the main argument in the

CALCIUM AND PHOSPHORUS BALANCES

WR. AGE 28 FEMALE UNIT NO 238476

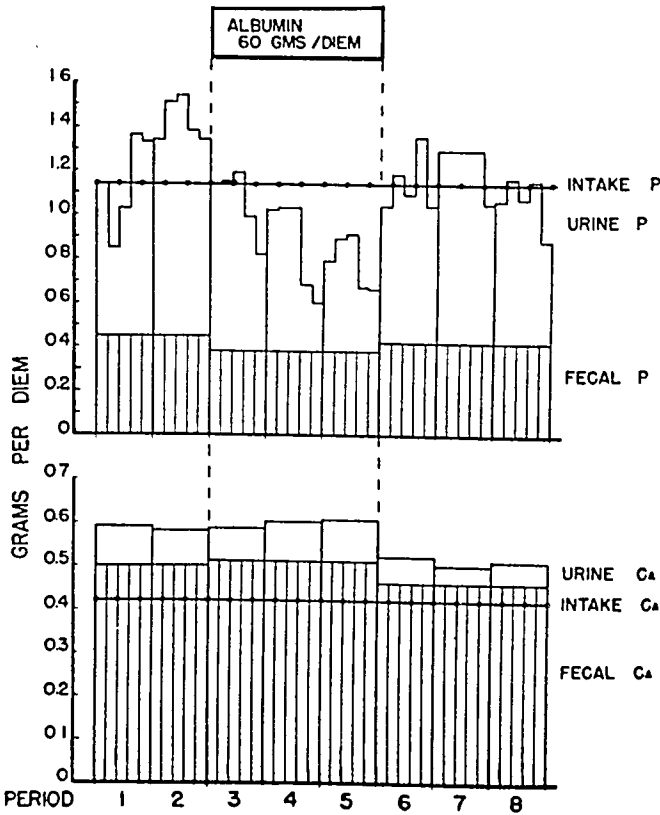


FIG. 11
For legend, see Figure 9.

CALCIUM AND PHOSPHORUS BALANCES

EUG. G. AGE 26 MALE UNIT NO. 188107

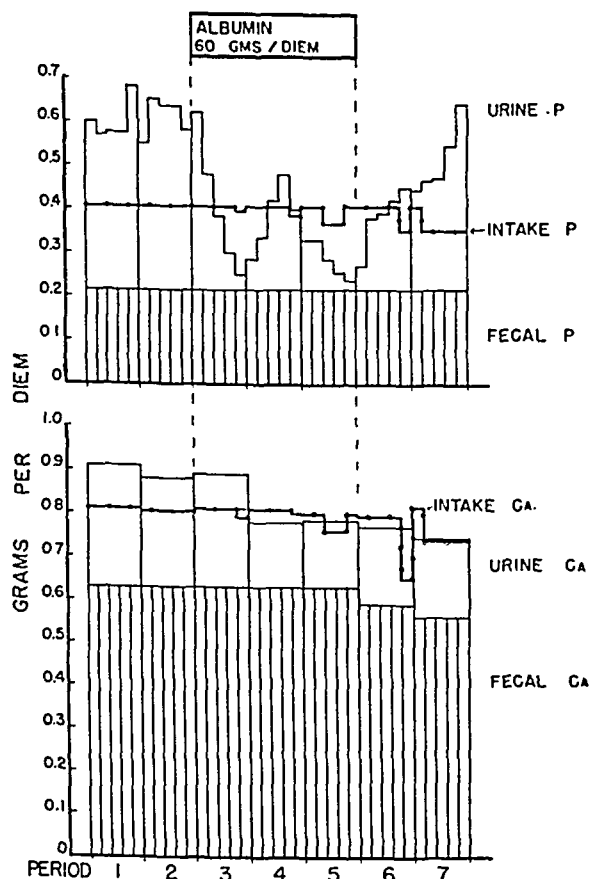


FIG. 12

For legend, see Figure 9.

contention that this patient was unable to convert more than a small portion of the injected protein into cellular protoplasm. The calcium of the urine became less in the experimental period. In the post-control period its excretion almost ceased.

The basic diet of W. R., who was in nearly normal health, corresponded rather closely to that of G. V. and was low in nitrogen and calcium but adequate in phosphorus and calories (Table I). Figure 11 shows the pronounced decrease in the urinary phosphorus and the positive phosphorus balance during the last ten days on albumin. The irregularity of the curve of urinary phosphorus is probably due to the fact that she received five different diets during the experimental period each of which contained the same amount of nitrogen, but differed somewhat in its content of cal-

cium and phosphorus. As the method of continuous sampling was used for the analysis of her diets, the only analytical values obtained for intake were those for a five-day period. The negative calcium balance was not unexpected in view of the suboptimal intake of this element. It is to be noted that urinary calcium decreased in the post-albumin control period. We have then, in this patient, essentially a duplication of the results previously described in G. V.

On analysis of the diet of Eugene G., it was found to contain only 0.421 Gm. of phosphorus, which was less than had been anticipated. Since the experiment was already well under way when this was discovered, no supplementary phosphate was given. Calculations had shown the diet to be deficient in calcium, and the deficit in dietary calcium was made up by the administration of calcium gluconate in divided doses so that the daily intake was slightly in excess of 0.8 Gm. With the injection of albumin and in the post-control periods, urinary phosphorus decreased to very low levels. Fecal phosphorus, on the contrary, did not decrease. Because of the very low phosphorus intake, the balance was only slightly positive even at the time when the urinary excretion was minimal. The small rise in phosphorus excretion in period 4 coincides with an intercurrent infection when the patient was mildly febrile and had a few diarrheal stools. Although the intake of calcium should have been sufficient to maintain equilibrium, the balance was rather consistently negative. (This may have been due to the fact that the limited supply of available phosphorus was all used to meet the demand for conversion of albumin to cell protein, thus leaving little or no phosphorus to combine with the calcium.) However, the urinary calcium was affected as in the other patients and decreased in the second and third periods on albumin and in the post-albumin control period.

This consistent decrease in the urinary calcium is not without interest. Albright (13) has noted the association of decreased urinary calcium with intravenous administration of plasma protein. From the present studies, it seems to be of little importance in its effect on the overall calcium balance and correlates poorly with some of the other phenomena observed. It occurs whether or not there is a decrease in urinary phosphorus

(data on E. G.) and does not relate directly to the proteinuria. Serum calcium was followed carefully in only one patient (Eugene G.) and in this instance it rose from a control value of 11.3 mg. per cent to 14.9 mg. per cent, coincidentally with the peak value for the concentration of serum protein. This appears to be an indication that calcium was bound to the albumin retained in the blood stream. Nevertheless, from the quantitative point of view, our data suggest a far greater actual retention (decreased urinary excretion) of calcium than was required by the extra albumin in circulation. It is true, of course, that we are unable to give an accounting for the albumin which has diffused into the lymph or extracellular fluid and which might be expected to bind calcium. That some of the injected albumin may be reconstituted in the form of calcifiable osteoid tissue is likewise a possibility (13).

Actual versus "theoretical" nitrogen balance

In the metabolism of protoplasm the exchanges of nitrogen must in the long run be associated

with exchanges of phosphorus and potassium in amounts comparable to the proportion which exists in tissue, if normal structural relationships are to be maintained. The essential correctness of this thesis has been repeatedly demonstrated by Albright *et al.* (23-25) and constitutes the basis for the computation of the theoretical nitrogen balance. If one corrects the phosphorus balance for the amount combined with calcium, the remainder should give an index of the amount of nitrogen required in the anabolism and catabolism of tissue. In practice, during metabolic experiments, the actual nitrogen balance and the theoretical nitrogen balance are in fairly good agreement during the oral feeding of protein and in fasting.

In the preparations of Figures 13-16, both the actual and theoretical nitrogen balances have been plotted, not as deviations from control periods, but as they actually occurred. This makes very little difference except in the case of Eugene G., where the balance of phosphorus in preliminary periods was consistently and significantly negative.

ACTUAL AND THEORETICAL NITROGEN BALANCE

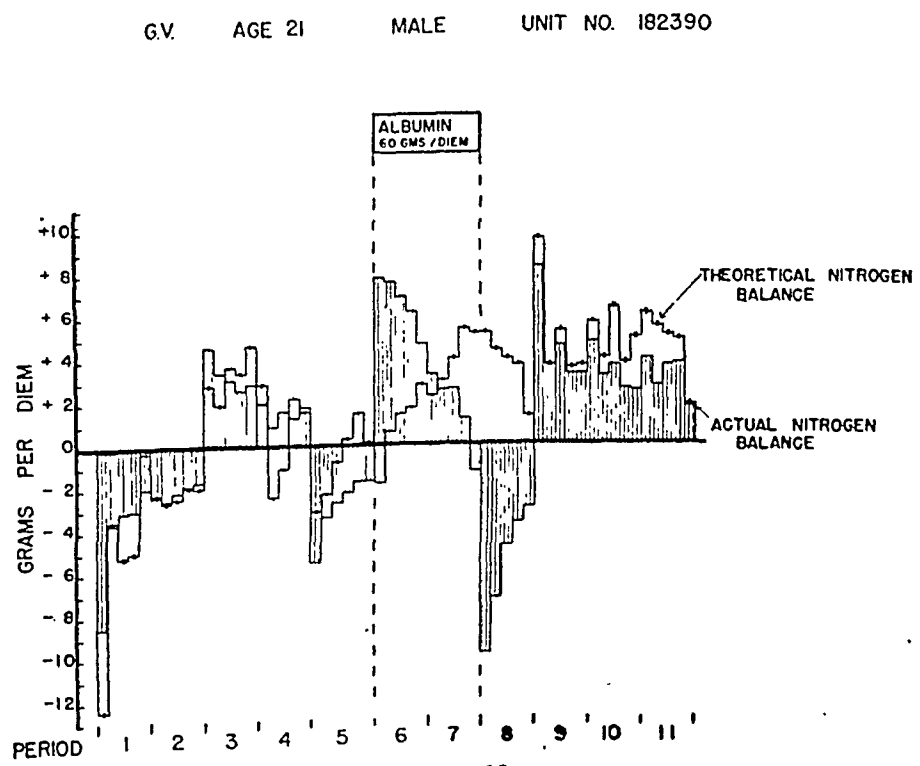


FIG. 13

The actual nitrogen balance is represented by the lined columns, the theoretical nitrogen balance by the clear columns. When overlapping occurs, the borders of the theoretical nitrogen balance columns are demarcated by squares superimposed on the limiting lines.

ACTUAL AND THEORETICAL NITROGEN BALANCE

EG AGE 43 FEMALE UNIT NO 267749

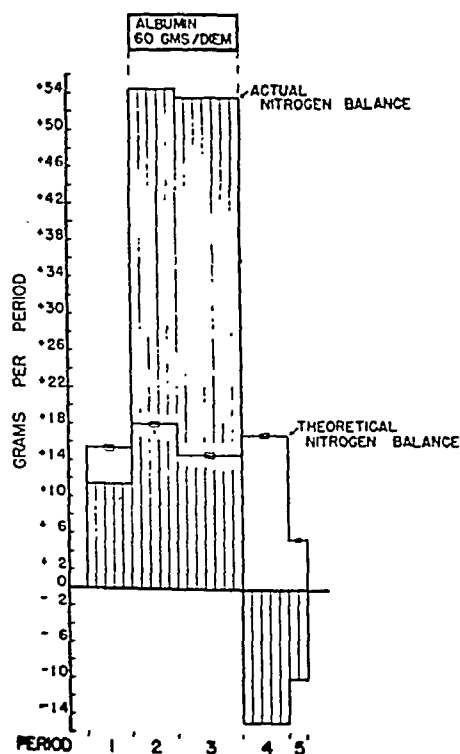


FIG. 14

For legend, see Figure 13.

In G. V. the actual and theoretical balances of nitrogen agreed fairly well until albumin was given. During these two periods the theoretical balance was at first much less positive than the actual balance, and in the post-albumin period it remained strongly positive although the actual balance had become negative (Figure 13).

The actual nitrogen balance of E. G. was markedly positive throughout the administration of albumin, but the theoretical balance was small and did not increase in the stepwise fashion observed in the other patients (Figure 14), a circumstance which will receive further comment presently.

The data on W. R. (Figure 15) are quite similar to those on G. V. and show a progressive increase in the "theoretical" retention of nitrogen during the 15 days on albumin. The theoretical balance differs from G. V., however, in that it did not continue to be strongly positive in the post-albumin period.

ACTUAL AND THEORETICAL NITROGEN BALANCE

W.R. AGE 28 FEMALE UNIT NO. 238476

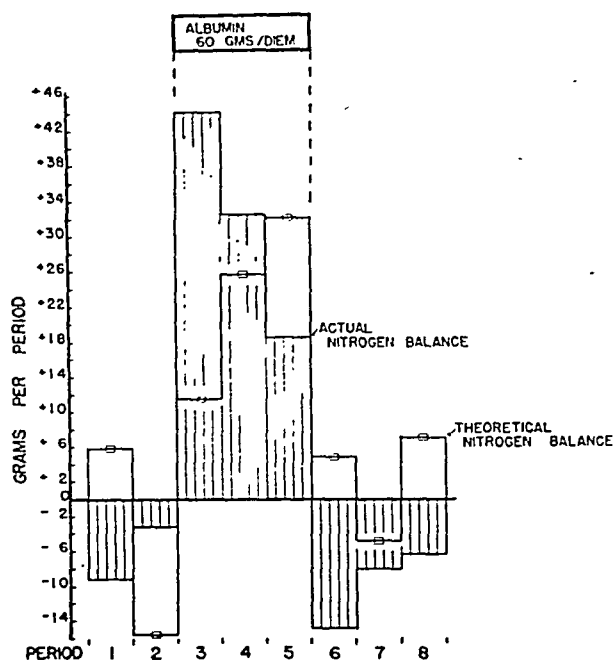


FIG. 15

For legend, see Figure 13.

ACTUAL AND THEORETICAL NITROGEN BALANCE

Eug. G. AGE 26 MALE UNIT NO. 188107

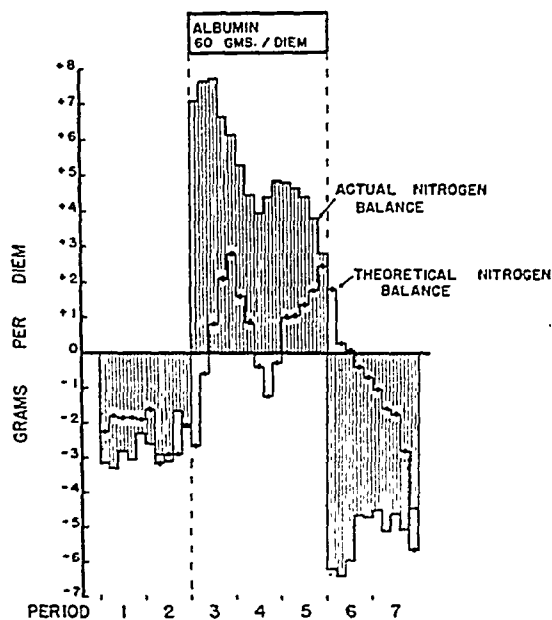


FIG. 16

For legend, see Figure 13.

The general trend of events in subject Eugene G. (Figure 16) was similar to that in G. V. and W. R., but one notes that the theoretical nitrogen balance is only slightly positive during albumin injections when computed from phosphorus accruing to the body. On the other hand, when calculated from the deviation of the phosphorus balance from that of the preliminary control periods, the theoretical balance of nitrogen is almost as great as in the other two patients. We have commented previously on the almost complete disappearance of phosphorus from the urine of this man as the administration of albumin was continued. This suggests that the phosphorus derived from endogenous sources, instead of being excreted as in the control period, was being reutilized. It is to be noted further that his intake of phosphorus was low and that there may have been an obligatory excretion of phosphorus in the feces, for the latter did not decrease even when the demand for phosphorus seems to have been marked. Thus, the whole trend of his response may have been altered by the limited quantity of available phosphorus.

The fate of the injected protein

We have used the "theoretical" nitrogen balance, as has Albright (13, 23), to estimate the conversion of phosphorus-poor albumin into cel-

DIAGRAM TO ILLUSTRATE METHOD OF ESTIMATING RETENTION OF ALBUMIN AND BURNING OF ALBUMIN

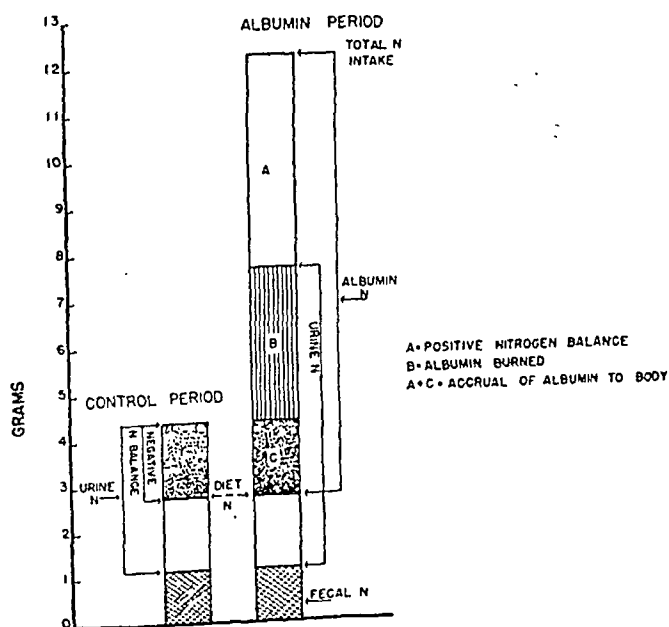


FIG. 17

CUMULATIVE CONVERSION AND BURNING OF ALBUMIN

G.V. AGE 21 MALE UNIT NO 18239Q

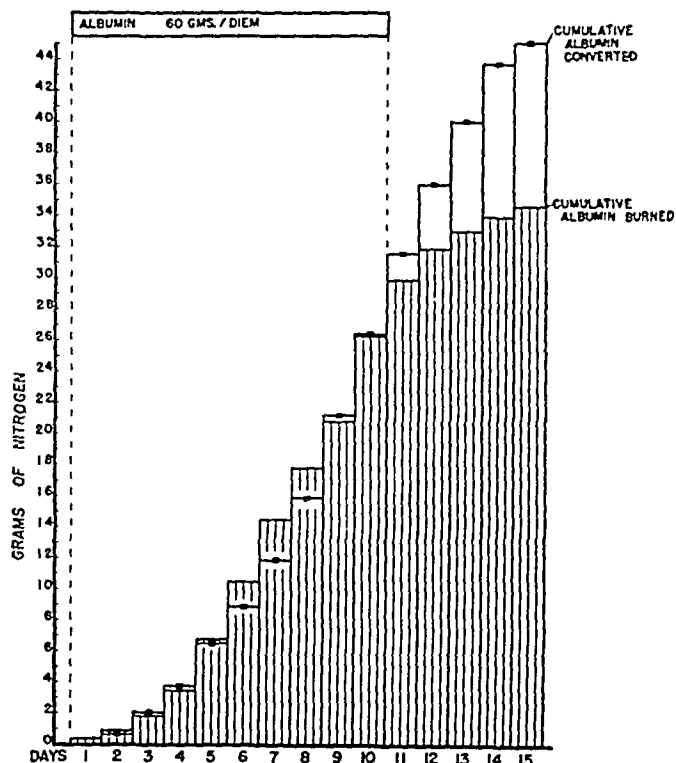


FIG. 18

Chart construction is such that the column for day 15 gives the sum of the metabolic effects from day 1 up to that moment.

The cumulative albumin burned is represented by lined columns; the cumulative albumin converted, by the total height of the clear columns.

When overlapping occurs, the limiting borders of the clear columns are demarcated by lines with squares superimposed on them.

lular protein associated with the average amount of phosphorus. The fraction of the injected albumin which has been catabolized (deaminated) may be estimated from the rise in the excretion of the urinary nitrogen above the baseline of the control periods. The fraction of the unchanged albumin wasted by excretion in the urine can readily be measured also. With this information at hand, a balance sheet can be drawn up which shows in a general way what has happened to the albumin at any moment in the experiment. The injected albumin can be roughly accounted for as follows:

- Albumin catabolized.
- Albumin converted to tissue protein.

- (c) Albumin remaining in the blood stream, extracellular fluid, etc.
 (d) Albumin excreted in the urine.

There are obvious limitations to these calculations. For example, one cannot tell whether albumin is converted to tissue protein and then catabolized, or whether it is directly degraded, nor can one follow the fate of the carbon residues of the deaminated protein. The exact location of albumin apparently retained as such in the body is not known except for that part remaining in the plasma.

In setting up the balances two main assumptions were made: (1) that the exchange of nitro-

CUMULATIVE BURNING AND CONVERSION OF ALBUMIN

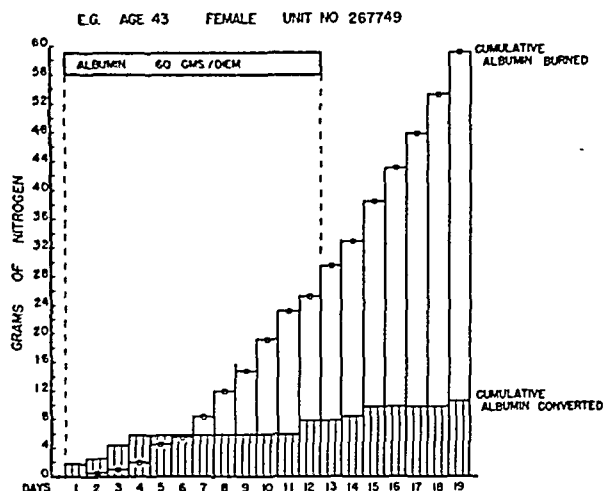


FIG. 20

The cumulative albumin converted is represented by lined columns; the cumulative albumin burned, by the total height of the clear columns from the baseline to the lines with squares superimposed on them.

CUMULATIVE RETENTION AND CONVERSION OF ALBUMIN TO TISSUE (?) PROTEIN

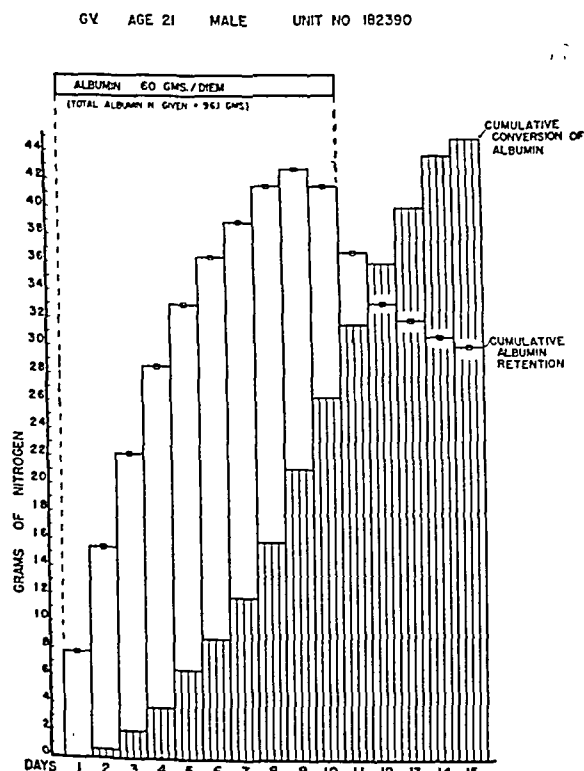


FIG. 19

Chart construction is such that the column for day 15 gives the sum of the metabolic effects from day 1 up to that moment.

The cumulative albumin converted is represented by lined columns; the cumulative albumin retained, by the total height of the clear columns, from the baseline to the lines with squares superimposed on them.

gen, phosphorus and calcium as measured on the final days of the control period would have remained constant throughout the experiment, had albumin not been administered; (2) deviations from the baseline of the control period could be ascribed to the albumin injections. Figure 17 has been constructed to illustrate the principles involved. The data are those obtained on G. V. The basic intake of nitrogen failed to meet the endogenous requirement of this patient, and he was in slightly negative balance during the foreperiod. When albumin was given, there was an increase in the urinary nitrogen above the basic excretion of the foreperiod. This represents albumin deaminated (burned). The gross retention of albumin in the body is the difference between that injected and that deaminated. The albumin retained is, therefore, greater than the actual positive nitrogen balance by this method of computation.

The cumulative burning of albumin in comparison with the amount converted to tissue protein in the case of G. V. has been graphed in Figure 18. One notes that neither process occurs to a significant degree until the third day of albumin injections. Thereafter both processes increased in stepwise fashion and continued even during the post-control period. The cumulative reten-

tion and conversion of albumin for this patient is depicted in Figure 19. There are two main points of interest, the delay in the conversion of

the retained albumin, and the apparent conversion of more albumin than was retained at the end of the control period. There are perhaps two possible explanations for the latter anomalous situation: (1) an error in the calcium-phosphorus balance which made the retention of the phosphorus appear greater than it actually was; (2) utilization of a small amount of the phosphorus for some other purpose than the conversion of albumin to tissue protein.

Figures 20 and 21 which record the cumulative conversion and burning, and cumulative retention

CUMULATIVE RETENTION AND CONVERSION OF ALBUMIN TO TISSUE (?) PROTEIN

E.G. AGE 43 FEMALE UNIT NO 267749

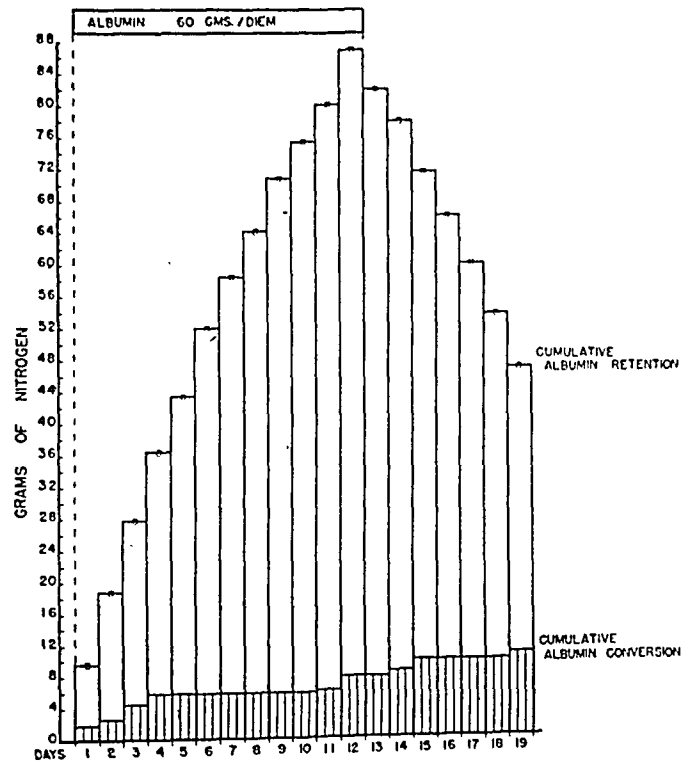


FIG. 21

For legend, see Figure 19.

CUMULATIVE BURNING AND CONVERSION OF ALBUMIN

WR AGE 28 FEMALE UNIT NO 238476

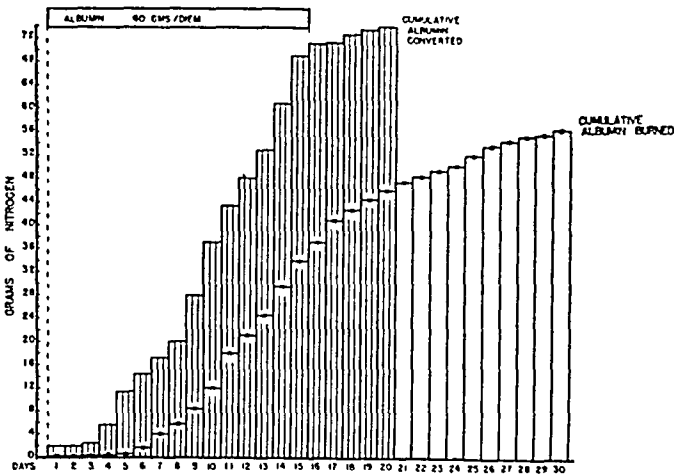


FIG. 22

For legend, see Figure 20.

CUMULATIVE RETENTION AND CONVERSION OF ALBUMIN TO TISSUE (?) PROTEIN

WR AGE 28 FEMALE UNIT NO 238476

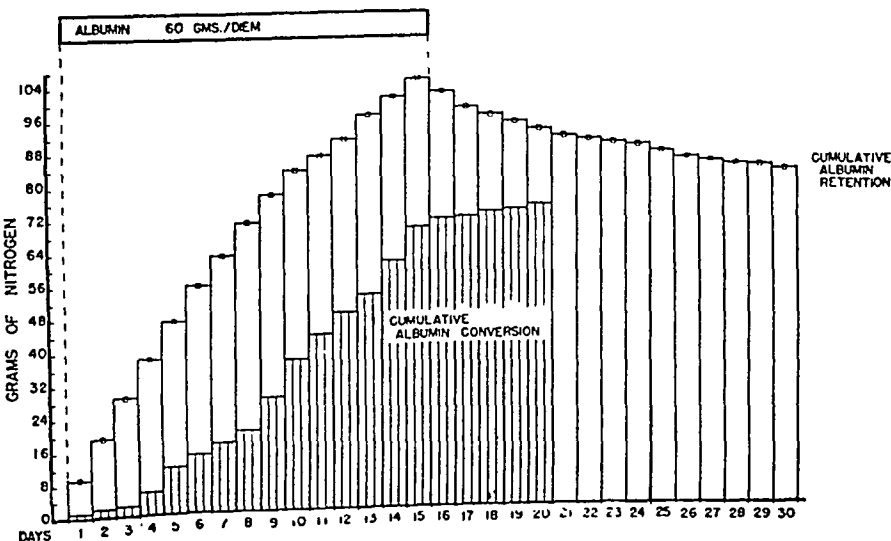


FIG. 23

For legend, see Figure 19.

CUMULATIVE CONVERSION AND BURNING OF ALBUMIN

E. G. AGE 26 MALE UNIT NO. 188107

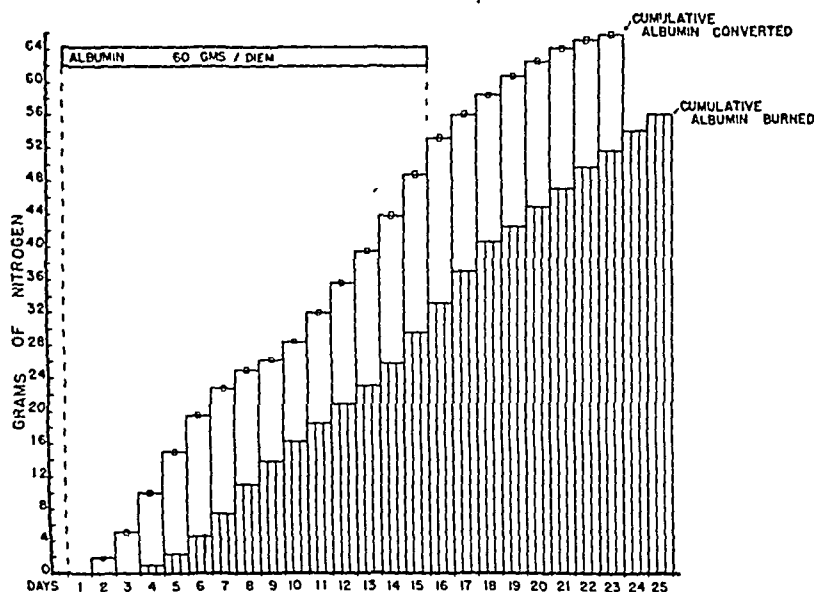


FIG. 24

For legend, see Figure 18.

and conversion of albumin, respectively, by E. G. should be contrasted with those of G. V. In E. G. deamination proceeded in somewhat the same fashion, but to a greater degree and the amount of albumin converted to cell protein was very small. Even at the end of the post-control period, a large percentage of it remained unchanged in the body. Figures 22 and 23 show the same processes in patient W. R. The deamination began slowly, while conversion was somewhat more rapid and did not appear to continue beyond the fifth post-control day. Figures 24 and 25 prepared from the data on Eugene G. are similar. Evidence of conversion could no longer be obtained after the seventh post-albumin day. The catabolism occurred more slowly, and the urinary nitrogen was still above the pre-albumin level ten days after the injections were discontinued. About 50 per cent of the injected protein was incorporated into cell protein if one computes this fraction from the deviation of the phosphorus balance from the initial control periods.

The amount of albumin retained in the plasma of the individual patients at different times is presented in Table II. Inasmuch as these values are admittedly rough estimates, no definite con-

CUMULATIVE RETENTION AND CONVERSION OF ALBUMIN TO TISSUE (?) PROTEIN

E. G. AGE 26 MALE UNIT NO. 188107

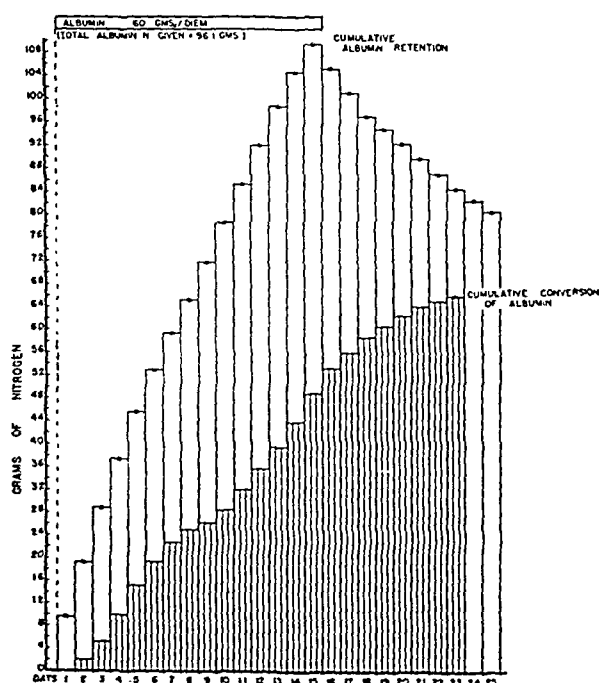


FIG. 25

For legend, see Figure 19.

TABLE II
Albumin retained in plasma

Subject	Day of experiment	Dose to date	Extra amount in plasma	As per cent total
		Gms.	Gms.	Gms.
G. V.	6	360	95	26
	9	540	184	34
	15	540	65	12
E. G.	5	300	43	14
	8	480	93	19
	12	720	117	16
	15	720	108	15
W. R.	3	180	40	22
	12	720	112	16
	14	825	154	19
	23	885	96	11
Eugene G.	4	240	45	19
	9	540	94	17
	15	900	213	24
	19	900	93	10
	23	900	58	6

Note: Many of the calculations of plasma volume were made from changes in the hematocrit alone, according to the formula $PV_2 = PV_1 \frac{(H_1)(100 - H_2)}{(100 - H_1)(H_2)}$. Because of the known inaccuracies of venous hematocrit determinations, these figures are assumed to be rough estimates.

clusions will be drawn. However, it was our impression that the albumin seemed to leave the blood stream most rapidly in G. V. who had the smallest dose and the most pronounced proteinuria and least rapidly in E. G. who utilized it poorly. In no instance were observations continued long enough for all the extra albumin to have disappeared.

A summary of the fate of the albumin administered to these patients is given in Table III. The large excretion of urinary protein in G. V. and the comparatively small conversion of albu-

TABLE III
Summary of albumin administration
(As per cent of the amount given)

Subject	Male		Female	
	G. V.	Eugene G.	E. G.	W. R.
Albumin burned	35.9	39.0	51.2	39.9
Albumin excreted in urine	26.6	5.3	8.3	1.6
Albumin retained in body	37.5	55.7	40.5	58.5
Albumin converted to tissue protein	100.0	100.0	100.0	100.0
	46.6	45.6	9.2	52.4

min to tissue protein in E. G. are emphasized again. W. R. and Eugene G., who were essentially normal, reacted to the injections with remarkable similarity.

V. DISCUSSION

The clinical response to human serum albumin given intravenously depends not only on its nutritive properties as a protein, but also on two other factors, namely, its potent osmotic activity and its nativity to the human body. Probably because of the last-named factor it is metabolized comparatively slowly under normal circumstances. Therefore, when the substance is given in large quantities, its slow utilization results in the accumulation of significant amounts of osmotically active protein. Thus to predict the response of the individual to injections of human albumin, one should take into consideration (a) his state of nutrition, especially with respect to protein, (b) the permeability of the capillaries in general and of the glomerulus in particular, and (c) the status of the cardiac reserve. Once an individual is saturated with albumin the compensatory adjustments may break down and proteinuria, cardiac decompensation, or edema may occur. Each of our four patients reacted differently to similar daily doses of intravenous albumin, and the importance of pre-existing disease (E. G.), renal permeability to protein (G. V.), dietary intake (E. G. and Eugene G.) and cardiac reserve (W. R.) are clearly shown.

The nutritive response as determined by the nitrogen balance deserves some comment even though this has been well described by other authors (12, 26). A positive nitrogen balance was present in all our patients at all times during the administration of albumin with the one exception of G. V. at a time when proteinuria was excessive. This exception should perhaps be emphasized since it is perfectly clear that the escape of albumin in the urine may reverse the nitrogen balance at a time when the need for protein still exists and the patient is still capable of storing considerable amounts of nitrogen. Moreover, the significance of the positive nitrogen balance should be determined in the light of the following facts. There is abundant evidence that plasma proteins will pass certain membranous barriers (27, 28). Albumin given intravenously

escapes through the capillary walls into the lymphatic spaces and to a smaller extent into the interstitial spaces. The accumulation of the whole protein in plasma, lymph, and the interstitial fluid has been described by Riegel (26) as a metabolic dead space and this is true in the sense that the nitrogen retained in the acute experiment has not all been converted into cellular protein. Thus under only one condition could the positive nitrogen balance be used as an indication of the nutritive value of this protein; namely, when an accurate calculation of the unchanged albumin in the body is at all times available. The amount retained in the plasma can be determined comparatively easily by plasma volume and serum protein determinations, but the quantity held outside the plasma would require measurement of the concentration of the albumin in the extracellular fluid as well as the volume of the latter. Indirect calculations of the unchanged albumin from data such as ours are perhaps possible but can be done only if other balances (calcium, phosphorus and potassium) are carried out.

The mode, extent and determining factors of utilization of parenterally administered plasma protein has been of considerable interest both to us and to other investigators. If one accepts the hypothesis that retention of phosphorus with albumin is a sign that the latter is being converted to cell protein, it seems certain that many patients will be able to effect such a modification. Three of our four patients showed evidence of conversion. The failure of our fourth patient, E. G., certain postoperative patients (26), and one normal patient who was receiving only 37.5 Gms. of protein a day (29) to show evidence of significant utilization may be taken as evidence that albumin is subject to the general laws of protein metabolism in the body.

The efficacy of albumin in relation to food protein was studied in only one patient, G. V. Beef protein orally and albumin intravenously were compared in quantities containing nearly equal amounts of nitrogen. The total conversion of albumin to cell protein by our method of estimation was 46 per cent, that for beef protein 22 per cent. It is obvious that no conclusions can be drawn from a single trial of this nature.

The actual mode of utilization of parenteral plasma protein has been discussed by several groups of workers. Whipple and his associates (28) have postulated "fluid interchange between the reserve stores and organ cells in which the protein is produced, modified and utilized in the body economy." Such a give and take might be accomplished without breaking the protein into its constituent amino acids. An alternate explanation is offered by Eckhardt (12) who suggests that the process is one of gradual decay, and that only as the protein is split into its individual components does it become available to the cells. According to this hypothesis albumin introduced into the blood stream would be broken down slowly by proteolytic enzymes and those constituents not of immediate use for re-synthesis of protein would be deaminated and excreted. By observing the rate at which injected albumin disappeared from the plasma, he was able to fit his data to a standard decay curve with a 50 per cent disappearance time in five days. Justification for neglecting the albumin held in extracellular fluid and lymph is based on the assumption that the proteins in these systems are in equilibrium with the plasma, although not necessarily of equal concentration. Thus when one measures the disappearance from the blood, one also measures the rate of disappearance from the extravascular spaces.

We were unable to establish similar decay curves in our patients because of too infrequent determinations of plasma volume. Decay in the sense used by Eckhardt would in our patients include albumin burned and converted, if one neglects the albumin lost in the urine. We have computed decay in our subjects using a time of five days for 50 per cent disappearance. Specifically this means that we have assumed that by the end of a five-day period, 50 per cent of the injected albumin would no longer be present as such in the body, but would have been either degraded or converted to cell protein. The values obtained for decay are compared with the sum of burning and conversion in Table IV and are expressed cumulatively as grams of nitrogen. The formula used for computing decay is that given for a series of daily injections (12). One sees fair correlation in the three patients, G. V., W. R., and Eugene G. This positive correlation we be-

TABLE IV.
Calculated decay of albumin vs sum of albumin catabolized and converted

Subject	G. V.		E. G.		W. R.		Eugene G.	
	Calculated decay	Sum of catabolism and conversion	Calculated decay	Sum of catabolism and conversion	Calculated decay	Sum of catabolism and conversion	Calculated decay	Sum of catabolism and conversion
5 days	15.7*	13†	15.7*	11†	15.7*	12†	15.7*	17.6†
10 days	47.5	52	47.5	25	47.5	42	47.5	45
15 days	72	80	90.5	48	87.5	102	87.5	78
20 days			95.5	72	116	119	116	107
25 days					130	126	130	121

* Values in this column refer to the per cent of decay that had taken place by the 5th, 10th, 15th day, etc.

† Values in this column refer to the sum of catabolism plus conversion also expressed as per cent of the amount of albumin received by the patient up to and including the 5th, 10th, and 15th days respectively.

lieve adds further support to the use of the theoretical nitrogen balance as a means of determining the conversion of albumin to some other form of protein. It is also clear that the rate at which a standard dose of albumin is metabolized is closely similar in these three individuals.

The poor correlation in E. G. is in agreement with our other data which indicate that a considerable fraction of the injected albumin remained as such presumably in edema fluid. Partial confirmation of this is found in the fact that the protein concentration of the pleural fluid was 6.2 Gms. per cent. It seems reasonable that excessive quantities of albumin leaked into the interstitial fluid in this patient, possibly because of increased capillary permeability. However, it does not seem likely that stagnation of the protein in the extracellular spaces was purely on a mechanical basis for if conversion of the albumin to cell protein and hence its removal from interstitial fluid depends upon contact with cells bordering on extravascular spaces, there should have been no lack of opportunity for this to occur. One must not overlook a possible influence of diet which was perhaps critically low in calories (Section I). The synthesis of protein in the cells may have been nearly maximal before the albumin was given, and further increase was prevented by a lack of source of energy.

The mechanism of the proteinuria, which eventually occurred in all our patients, requires brief comment. In no case was there suspicion of serious renal damage, since values for glomerular filtration rate and renal plasma flow were within normal range. This does not, however, rule out

the possibility of abnormal glomerular permeability, especially in G. V. who had traces of protein in his urine prior to albumin injections. It might be argued (a) that injections of albumin damage the glomeruli and that leakage of protein is permitted. A previously damaged glomerulus would perhaps leak more protein than one which was formerly normal. (b) That the normal glomerulus permits the passage of small amounts of protein which is ordinarily recaptured by the tubular cells and that proteinuria after albumin is secondary to the high concentration of albumin in the plasma and is due to filtration of more albumin than can be reabsorbed by the tubular cells. In view of the recent observations of Monk and Yuile (30), Terry *et al.* (31), and Rather (32) and the arguments advanced by them, the second mechanism appears to fit the facts better than the theory of damage. As was the case in animal experiments (31), the albuminuria of our patients ceased soon after the injections were stopped and tests of kidney function showed no decrease as a result of the procedure.

VI. SUMMARY

Each of four subjects received a daily dose of 60 Gms. of concentrated sodium-free albumin for ten or more days. Balances of nitrogen, calcium, phosphorus, and potassium were made during control, albumin, and post-albumin periods. Concomitant observations of serum protein fractions, plasma volume and renal function were made.

The individual variations in response are noted, namely (1) the development of an intense proteinuria in a patient convalescent from rheumatic

fever, without evidence of impaired renal function; (2) the retention of water with peripheral edema, hydrothorax and pericardial effusion, and the poor utilization of the injected protein in a woman with probable generalized vascular disease; (3) the excellent utilization, but eventual development of signs of cardio-respiratory embarrassment in a young woman in fair health except for moderate undernutrition; and (4) the modification of the response in a normal young male by other dietary factors. The mechanisms of these reactions are discussed.

It is felt that proteinuria will develop eventually in any patient when saturation with albumin has occurred and that this is probably not due to renal damage, but represents a physiological response when the maximum reabsorptive capacity of the renal tubules for protein is exceeded.

ACKNOWLEDGMENT

The authors express their appreciation to Dr. Lawrence A. Kohn, Department of Medicine, University of Rochester, for the determinations of plasma and blood volumes, and to Dr. Eric L. Alling, Department of Pathology, University of Rochester, for analysis of the serum proteins by electrophoresis.

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CONGENITAL METHEMOGLOBINEMIA. A CLINICAL AND BIOCHEMICAL STUDY OF A CASE¹

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Congenital methemoglobinemia is an unusual condition in which a large amount of intracellular hemoglobin exists as methemoglobin—pigment in which the hemoglobin iron is in the ferric state and incapable of carrying oxygen. The congenital type of methemoglobinemia is present from birth and is characterized by the constancy of the level of methemoglobin, by the relatively mild associated symptoms, and by polycythemia with no evidence of excessive blood destruction. There are 15 verified cases of congenital methemoglobinemia in the literature (1–10) and 10 additional cases of idiopathic methemoglobinemia (11–15) in which definite information as to the congenital aspect was lacking. In all of these cases the abnormal pigment was identified spectroscopically.

The subject of this study was a young man who presented the typical clinical picture of congenital methemoglobinemia. We have now followed him for five years—sufficient time to evaluate different forms of therapy for this condition.

CASE HISTORY

F. S., a 23-year-old public accountant, was admitted in December 1942 because of persistent cyanosis. He was born a "blue baby" and had been continually cyanotic with no noticeable fluctuation of degree of cyanosis during all his life. He could not indulge in strenuous exercise because of dyspnea, easy fatigability and pounding temporal headaches. He took no drugs and had no gastro-intestinal symptoms and there was no family history of similar cyanosis.

Physical examination disclosed a well-developed young man, 5 feet 11 inches tall and weighing 207 pounds. His skin had a slate blue color which was especially apparent in his lips, ears and finger nail beds. His conjunctivae were congested and the veins in the optic fundi were dark and dilated. There were no abnormalities on examination of his heart and lungs. Blood pressure was

130/90. He had no clubbing of his fingers, and his spleen and liver were not palpable.

Laboratory data: Hinton test for syphilis was negative; RBC was 6.5 million per mm.³; hematocrit was 58 per cent; Hb was 19 gms. per 100 cc.; WBC was 7,850 per mm.³ with a normal differential count; reticulocytes were 2.2 per cent. Hypotonic saline fragility was normal. Bone marrow puncture showed only a normoblastic hyperplasia, consistent with his polycythemia. Urine and stool examinations were negative. Plasma proteins were 7.8 gms. per 100 cc. with 4.4 gms. of albumin; serum bilirubin was 0.5 mgm. per 100 cc. Venous pressure was 100 cm. of water; circulation time was 14 sec. (decholin); vital capacity was 4 liters. Bromsulfalein excretion test showed a retention of 5 per cent of the dye in the serum in 30 min. EKG was normal; BMR was -15 and -12 per cent. X-ray of the chest showed normal lung fields with a heart of normal size and shape. X-ray of the large bowel showed no abnormalities. Spectroscopic examination of the blood showed the absorption bands characteristic of methemoglobin. By difference between total pigment and oxygen capacity it was estimated that 45 per cent of the total blood pigment was in the form of methemoglobin. Subsequent determinations of methemoglobin were done by the colorimetric method of Evelyn and Malloy (16). This method was checked by the carbon monoxide capacity method of Van Slyke and Hiller (17) and found to be accurate to ± 0.2 gm. in our hands.

After a control period of one week in which the level of methemoglobin remained constant he was given 90 mgm. of methylene blue intravenously. In 10 minutes half of the methemoglobin disappeared and by 30 minutes over 95 per cent had disappeared. He was then able to exercise vigorously without headaches and breathlessness which had previously so limited his activity. During the course of the following five years he was treated with ascorbic acid and with methylene blue by mouth. It was found that 240 mgm. of methylene blue in enteric coated tablets per day were well tolerated and were sufficient to keep him free of cyanosis. Coincident with this treatment polycythemia and reticulocytosis subsided. No toxic effects from this long-sustained treatment have been observed.

The identity of the abnormal pigment in this case was established spectroscopically as methemoglobin. There was a well-defined absorption band at 630μ which disappeared after addition

¹ Read at the Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 5, 1947.

² Fellow of the Nutrition Foundation. Present address: The Rockefeller Institute for Medical Research, New York, N. Y.

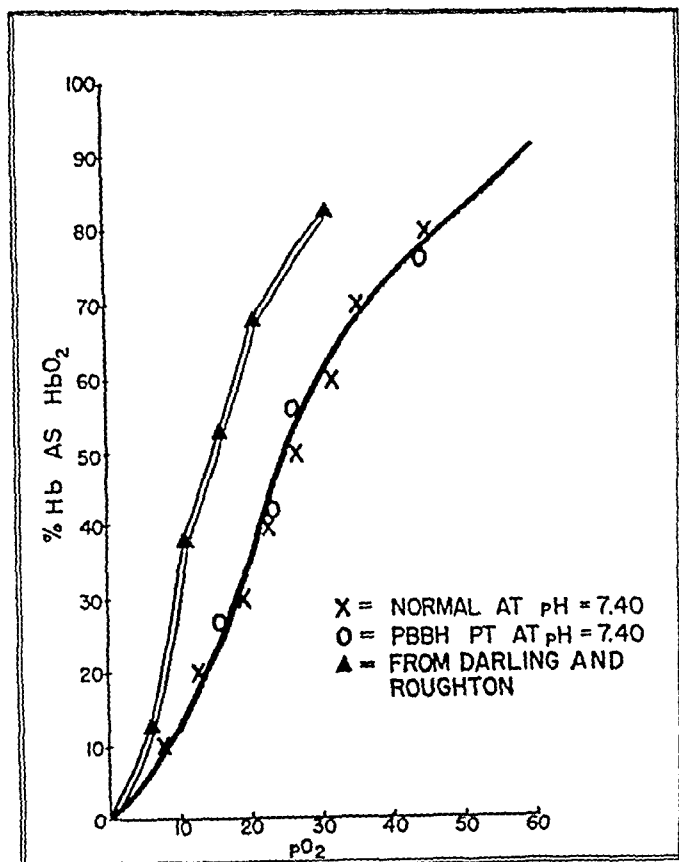


FIG. 1. OXYGEN DISSOCIATION CURVE OF PATIENT WITH 30 PER CENT METHEMOGLOBIN SUPERIMPOSED ON NORMAL OXYGEN DISSOCIATION CURVE

This is compared with the curve of Darling and Roughton on blood also containing about 30 per cent methemoglobin.

of sodium cyanide or of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) *in vitro*, or after injection of methylene blue *in vivo*.

Symptoms present in our patient and in others reported were those of anoxia due to the decreased oxygen-carrying capacity of the blood. In addition to this, Darling and Roughton (18) have recently described an additional adverse effect of the presence of methemoglobin on oxygen transport; this is a shift to the left of the oxygen dissociation curve as also occurs in carbon monoxide poisoning. This change hinders the transfer of oxygen from the blood to tissue.

The oxygen dissociation curve on the blood of our patient when his methemoglobin level was 30 per cent was determined by us and by the Harvard Fatigue Laboratory (Figure 1). In both cases the points fell on the normal curve and showed none of the changes described by Darling and Roughton. In four other cases in which oxygen dissociation curves were made, one (12) showed no shift while three showed definite shifts of the curve (1, 14, 19).

STUDIES OF THE MECHANISM

I. Site of the defect

To ascertain whether the factor responsible for the high methemoglobin content of the erythro-

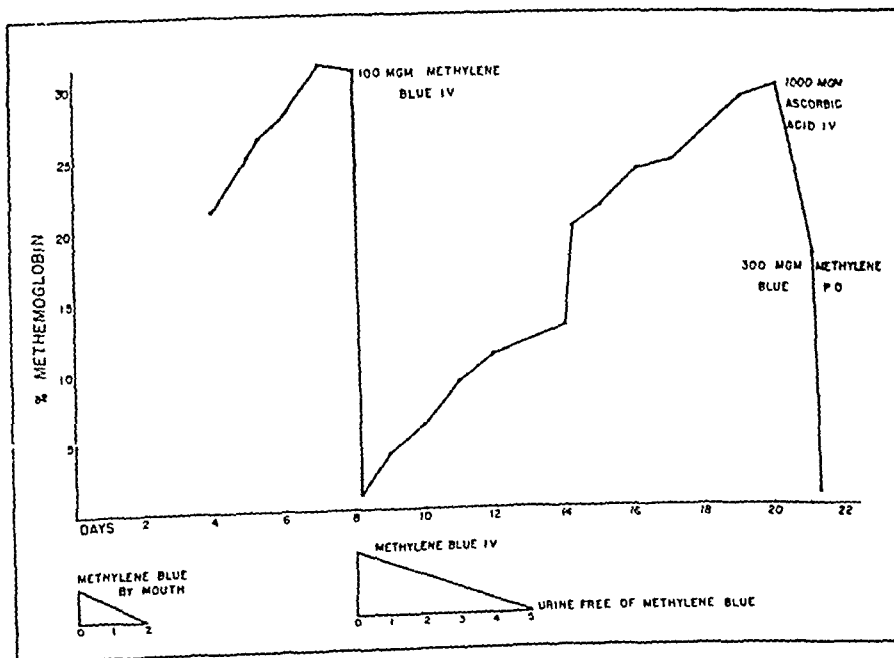


FIG. 2. RATE OF METHEMOGLOBIN FORMATION IN PATIENT F. S. AFTER CESSATION OF METHYLENE BLUE THERAPY

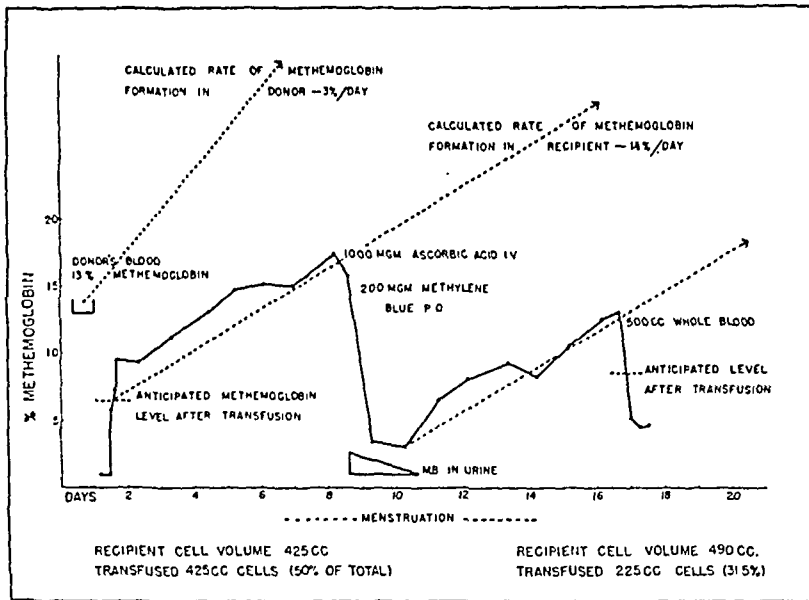


FIG. 3. BEHAVIOR OF BLOOD FROM PATIENT F. S. AFTER TRANSFUSION INTO A PATIENT WITH REFRACTORY ANEMIA

cytes was in the cells or the plasma the patient's red cells were incubated with compatible normal serum, and normal cells were incubated with the patient's serum. At the end of 24 hours in the former the methemoglobin was not significantly reduced, and in the latter methemoglobin was not produced. This confirmed previous *in vitro* observations that there was no plasma factor of importance (2, 10).

To ascertain whether there was any factor in the body, outside the red cells, that influenced methemoglobin formation in the cells, the following *in vivo* experiment was performed. The methemoglobin in the patient's circulation was changed to normal hemoglobin by methylene blue administration, and the subsequent rate of methemoglobin formation in the patient was observed for 13 days (Figure 2). There was an average daily transformation of 3 per cent of the total hemoglobin into methemoglobin. At a time when methylene blue therapy had changed the patient's methemoglobin nearly all to normal hemoglobin he was bled and 225 cc. of his erythrocytes were infused into a patient with refractory anemia (Figure 3). This subject was chosen as a recipient because there would be no new hemoglobin formation to complicate the experiment. The volume of cells infused was calculated to be exactly equal to the volume of the recipient's own

circulating erythrocytes, as estimated from determination of her plasma volume (T-1824 method) and her hematocrit. After the infusion the rate of methemoglobin formation per day was 1.5 per cent of the total hemoglobin. It therefore appeared that the infused cells were forming methemoglobin in the recipient at the same rate at which they formed it in the donor. This result makes it appear improbable that there were any abnormal factors outside the red cells of the methemoglobinemic patient that influenced the formation of methemoglobin.

II. Failure of methemoglobin reduction

A solution of hemoglobin obtained by laking human red cells when exposed to an atmosphere of 95 per cent air and 5 per cent carbon dioxide will be completely converted to methemoglobin in about five days (Figure 4). This was equally true of hemoglobin from normal human subjects and of hemoglobin from our patient. Intact red cells possess a mechanism which prevents this from occurring and maintains the hemoglobin iron in the ferrous state. When we incubated normal cells in the rocker perfusion apparatus (20) no methemoglobin was formed as long as glucose was present in the media (Figure 4). When sodium nitrite was added to normal cells under these conditions methemoglobin was formed and then

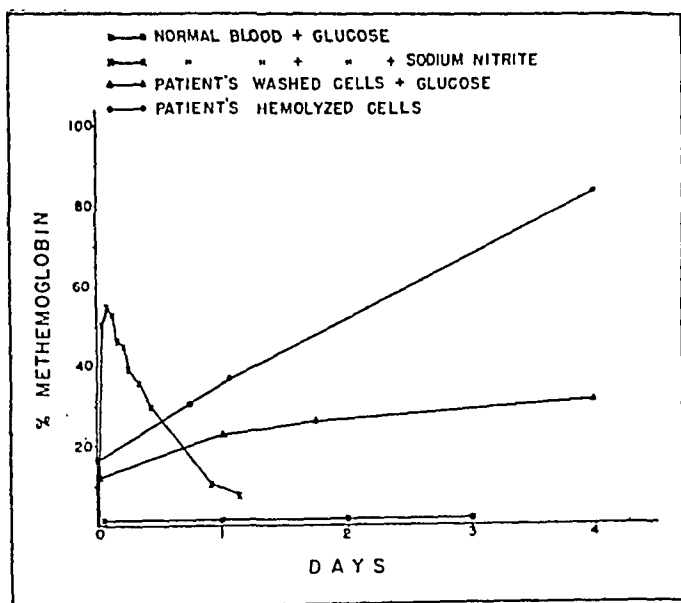


FIG. 4. METHEMOGLOBIN LEVELS AFTER *IN VITRO* INCUBATION OF HEMOLYZED CELLS AND INTACT CELLS IN THE ROCKER PERFUSION APPARATUS

The *in vitro* effect of sodium nitrite on normal cells is also shown.

completely reduced within 12 hours. When cells from our patient containing methemoglobin were incubated in the apparatus there was no reduction and the methemoglobin level rose to about 30 per cent. This inability of the patient's cells to reduce methemoglobin corroborates previous *in vitro* observations (2, 6, 10).

Studies of the *in vivo* reduction rates of methemoglobin were performed by injecting, intravenously, 0.5 gm. of sodium nitrite into normal individuals and into the patient. Nitrite was chosen because it rapidly produced methemoglobin and was itself entirely gone from the blood stream by 60–90 minutes (21). In both the normal subject and the patient the sodium nitrite caused formation of about 2.5 gms. of methemoglobin per 100 cc. of blood within 40 minutes. On the other hand, after three hours, half of the methemoglobin formed had disappeared in the normal subject while in the patient the methemoglobin level showed no tendency to fall even after seven hours (Figure 5). This indicates that the normal mechanism for methemoglobin reduction fails to operate in this patient.

III. The methemoglobin reduction mechanism of the erythrocyte

In vitro studies of methemoglobin reduction in the red cell have shown that this is an enzymatic process in which glucose and lactate act as hydrogen donors (22–24). In view of this relationship, the carbohydrate metabolism of our patient's erythrocytes was investigated. The resting oxygen consumption measured in the Warburg manometric apparatus of 2 ml. of the patient's cells

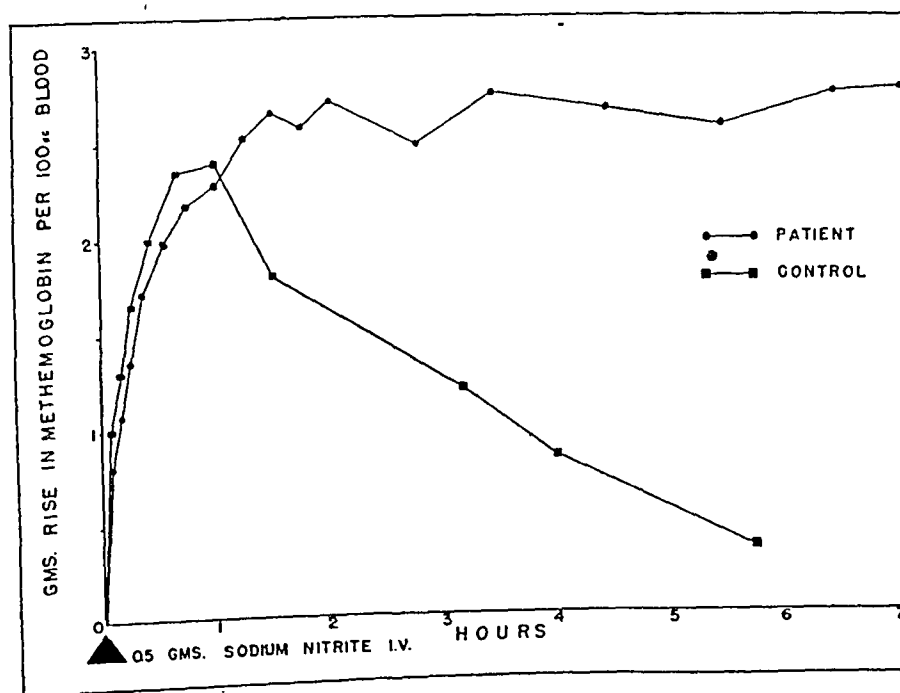


FIG. 5. EFFECT OF 0.5 GM. SODIUM NITRITE ON PATIENT F. S. AND ON A NORMAL SUBJECT

TABLE I

Chemical survey of factors involved in carbohydrate metabolism in patient's blood

	Glucose utilization	Lactate utilization	Whole blood inorganic P	RBC				
				15 min. hydrolysable P	Acid soluble P	Total P	Resting O ₂ consumption	Flavine adenine dinucleotide
	mgm. per cent per hour	mgm. per cent per hour	mgm. per cent	mgm. per cent	mgm. per cent	mgm. per cent	mm. ³ per hour per 2 cc. of cells	gamma per cent
Normal average range	10-20(28)	0-5	2.4-3.5(29)	8.1-10.5(30)	50-60(27)	47-114(29)	10-30	35
Patient	21.5 22.2	0-4	2.5 3.2	12.2 13.2	56.4 58.6	112.7 121.6	20	35

was 22 mm.³ in 30 minutes as compared with 19 mm.³ in the normal control. Glycolysis as measured by glucose utilization (25) and lactate formation was normal. There was no lactate utilization. Flavine adenine dinucleotide in red cells was measured by the method of Ball *et al.* (26) using the enzyme system of d-amino acid oxidase with d, l-alanine as substrate.³ The amount of enzyme present was the same as in a normal control. Values for the 15 minute hydrolysable P of which the chief component is adenosine triphosphate were well within the normal range. Acid soluble P values comprising largely glycerol phosphate (27) were normal. Measurement of the other P partitions also showed nothing which indicated any abnormality of phosphorylation. These findings are summarized in Table I. To the extent of these studies we were unable to find any defect in the carbohydrate metabolism of the erythrocyte.

IV. Action of methylene blue

Harrop and Barron in 1928 (31) demonstrated that the addition of methylene blue caused a ten-fold increase in the oxygen consumption of mammalian red blood cells. Such an increase occurred to the same degree in our patient's erythrocytes as in the normal erythrocytes. This increase does not occur when the cells are washed free of glucose. When blood from the patient with congenital methemoglobinemia was treated with methylene blue in the presence of 100 mgm. per cent glucose there was complete reversion of the methemoglobin present to active hemoglobin. This

reversion was inhibited by M/300 iodoacetate.

The beneficial therapeutic effect of methylene blue has been observed clinically for 15 years in secondary types of methemoglobinemia (32-34) and has been recently reported in congenital methemoglobinemia (8, 14). The marked therapeutic effect of methylene blue in our patient suggests that it replaces some constituent of normal cells that is necessary for the enzymatic reduction of methemoglobin and that this is lacking in the cells of our patient.

V. Other oxidizing and reducing substances

Since hydrogen peroxide is formed in certain enzymatic reactions and then is decomposed by intracellular catalase, it seemed possible that a deficiency of catalase could be responsible for the presence of methemoglobin. Accordingly the catalase content of the patient's erythrocytes was determined by a modification of the methods of Morgulis and Juszat (35, 36) using 0.1 M hydrogen peroxide in 0.02 M phosphate buffer (pH 7.0) in a Warburg vessel. A normal catalase content of the red cells was found.

There are also substances present in blood cells and plasma that are capable of reducing methemoglobin. Ascorbic acid and glutathione are two such substances. This patient had blood levels of ascorbic acid averaging about 0.25 mgm. per cent compared to normal values of 1.5 to 2.0 mgm. per cent (37) despite adequate dietary ascorbic acid. The reduced glutathione level of the patient's whole blood was 20 mgm. per cent as compared to normal values of 40 mgm. per cent (38). One might consider that these substances represent a secondary line of defense of the body should the cell reconversion mechanism not func-

³ The standard flavine adenine dinucleotide and the d-amino acid oxidase was generously furnished by Dr. E. G. Ball.

tion. Certain circumstantial evidence indicates that this might be true. When hemoglobin or methemoglobin is injected intravenously in a dog an equilibrium point is reached at a methemoglobin concentration of 30 to 40 per cent of the total pigment (39, 40). By coincidence the 11 cases of congenital methemoglobinemia in which the per cent methemoglobin was reported showed the same equilibrium concentration.

When 2 gms. of ascorbic acid were given intravenously the methemoglobin level dropped from 25 to 15 per cent in ten hours. On doses of 300 to 500 mgm. of ascorbic acid per day for two weeks it was possible to lower the level of methemoglobin to 8–10 per cent of the total pigment. The sluggishness of this reaction and the fact that d-ascorbic acid which is physiologically inert is equally as effective as the naturally occurring isomer suggest that the effect of ascorbic acid is due to its direct reducing action on methemoglobin (41, 42) rather than to restoration of the normal enzymatic reduction mechanism.

DISCUSSION

Studies on this case have emphasized the normal mechanism by which the organism is able to maintain the hemoglobin-methemoglobin equilibrium within the red cell at about 99.6 per cent in favor of the ferrous form which is essential for transport of oxygen (43). There is undoubtedly steady formation of methemoglobin in the red cell, but the cells are capable of reducing the methemoglobin as soon as it is formed. In congenital methemoglobinemia we have shown that this mechanism fails to function, and it is pertinent to look further into the nature of this mechanism.

In 1930 Warburg (22) showed that methemoglobin formed in intact red cells could be reduced by the cells when glucose was added. This reduction of methemoglobin was accompanied by the disappearance of the glucose and the appearance of pyruvic acid. This effect could be inhibited by iodoacetate. Gibson (44) has recently shown that methemoglobin reduction can occur in the presence of fluoride, but there is accumulation of phosphoglycerate. These findings indicate that the reduction of methemoglobin is coupled with

glycolysis. In the glycolytic process diphosphopyridine nucleotide (coenzyme I) is reduced and is available for reduction of methemoglobin. (Red cells also contain triphosphopyridine nucleotide [coenzyme II] which becomes reduced by the direct oxidation of glucose through phosphogluconic acid.) Gutmann (45) has shown that reduced pyridine nucleotide will slowly reduce methemoglobin directly and this reaction can be accelerated by the addition of methylene blue. In normal cells it is likely that a flavoprotein which is present acts, as in other tissues, as a carrier between the pyridine nucleotide and the iron porphyrin. Little is known about the nature and properties of the flavoprotein in the red cell. Studies of Gibson have shown that in abnormal cells of his patients with congenital methemoglobinemia there is no defect in that part of the cycle concerned with formation of reduced pyridine nucleotide. Since glycolysis was shown to take place normally in the cells of our patient it is probable that reduced pyridine nucleotide is also formed normally in his cells. Since methylene blue has such a pronounced effect on reducing the methemoglobin in his cells it seems likely that the defect lies in these substances which catalyze the reduction of methemoglobin by reduced pyridine nucleotide. Gibson concluded that this defect in his patients was a deficiency of the flavoprotein, the coenzyme factor. In heart muscle this factor has been shown to be a flavine adenine dinucleotide (46). We analyzed for this substance using the d-amino acid oxidase as a test system and found it to be present in normal amounts in the cells of our patient. It is still a possibility that in his red cells this factor which is deficient is the flavine mononucleotide in which case the deficiency would not have been apparent by our method of analysis. It is also possible as both Kiese (47) and Gibson point out that the methylene blue does not function in a system already functioning actively (the coenzyme I system) but rather enters into a secondary sluggish system (the coenzyme II system) and converts it into a rapidly active system. Further studies are necessary to find out what flavoproteins are present in the red cell and whether they have the capacity to reduce methemoglobin directly or require other as yet unknown factors.

SUMMARY

A case of congenital methemoglobinemia was studied which demonstrated the following characteristics:

1. The level of methemoglobin was approximately 40 per cent of the total pigment and remained constant from day to day.
2. In the untreated patient there was a compensatory polycythemia and no evidence of excessive blood destruction. Alteration of the O₂ dissociation curve was not present.
3. Methylene blue and ascorbic acid were effective therapeutic agents.
4. The cause of the methemoglobinemia was shown to be the absence of a factor in the erythrocyte mechanism that in normal red cells rapidly reduces methemoglobin.

ACKNOWLEDGMENT

We should like to express our gratitude to Mr. Francis Stanley, the patient, without whose interest and cooperation these studies would have been impossible, and to Miss Katherine Hubbel for technical assistance.

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THE EFFECTS OF INDUCED MALARIA, ACUTE STARVATION AND SEMI-STARVATION ON THE ELECTROPHORETIC DIAGRAM OF THE SERUM PROTEINS OF NORMAL YOUNG MEN¹

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During the last ten years, the technique for the electrophoretic analysis of serum proteins, originally described by Tiselius (1), has been standardized and a large number of important conditions have been studied. Luetscher (2) has reviewed this work and pointed out that the common denominator of all disease processes studied has been a relative decrease in the albumin fraction. Both acute and chronic infections produce an increase in the alpha globulin fraction which is often accompanied by an increase in the gamma globulin fraction. Chronic noninfectious processes such as ulcer or cancer also are accompanied by an increase in one or both of the alpha globulin fractions. Chow (3) has demonstrated that there exists a negative correlation between the relative amounts of serum albumin and the combined alpha globulins in disease states such as tuberculosis, ulcer and malnutrition. It has been shown that the characteristic response to protein depletion in the dog (4, 5) is also a decrease in albumin and an increase in the alpha globulins. Since this same change occurs in disease Chow has quite properly raised the question as to whether it is a response to the disease state or whether it is the result of the protein depletion which is common to these diseases.

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Experiments in this laboratory on the effects of induced malaria, of acute starvation and of semi-starvation on human volunteers provided an opportunity to study these questions in controlled and uncomplicated situations. Data from these experiments which throw some light on the relationship of protein depletion to serum protein composition during disease and starvation form the basis for this report.

CONDITIONS AND MATERIALS

All the subjects were males between the ages of 18 and 34 years and were members of Civilian Public Service. A careful medical examination failed to disclose signs of disease or abnormality when the men were admitted to the laboratory dormitory.

Eleven of these men took part in a long-term dietary experiment in which caloric and thiamine intake were carefully controlled. These diets were adequate in all known dietary constituents. This group of men was subjected at different times to both the stress of malarial fever and five days when they received no food. Ample time was allowed between the starvation stress and the induction of malaria for the subjects to recover completely from the effects of going without food.

These volunteers were inoculated with the McCoy strain of tertian malaria and all of them developed the active disease. After five to eight paroxysms and an average of 193 degree-hours of body temperature above 101° F., the malarial fever was terminated with quinine sulfate. Further details of this work will be presented elsewhere (6). Blood was drawn with the men in the fasting state the day before inoculation and again at the end of the febrile period. Plasma volumes were determined on the same day using the blue dye, T 1824. The concentration of the dye was determined by extrapolation of values obtained at 20, 30 and 40 minutes after the injection of the dye. The plasma volumes were calculated according to the procedure of Gibson and Evans (7).

Four of the 11 men were also subjected to five days of simultaneous starvation and hard work. The regimen of activity was so designed that the men expended 4,500 Calories a day during the fast. During this period the men lost an average of 13.8 pounds or 9 per cent of the initial body weight. Similar experiments which lasted for a shorter period of time have been described previously (8). Blood samples were drawn the morning of

TABLE I

The electrophoretic components of the serum proteins before and after a bout of induced malaria

Protein Fraction	Per cent of Total Pattern				Grams per 100 cc. Serum				Circulating Proteins, grams			
	Before malaria	after malaria	Change	per cent Change	Before malaria	After malaria	Change	per cent Change	Before malaria	after malaria	Change	Per cent Change
Alb.	64.2	55.9	-8.3	12.9	4.38	3.79	-0.59	13.4	148.1	127.5	-21.6	14.5
Glob.	35.8	44.1	+8.3	23.2	2.44	2.97	+0.53	21.7	82.5	99.9	+17.4	21.0
Alpha 1	4.1	6.8	+2.7	65.9	0.28	0.46	+0.18	64.3	9.5	15.4	+5.9	62.1
Alpha 2	7.7	8.1	+0.4	5.2	0.53	0.55	+0.02	3.7	17.9	18.5	-0.6	3.3
Beta	11.4	13.7	+2.3	20.2	0.77	0.93	+0.16	20.7	26.1	31.3	+5.2	20.0
Gamma	12.7	15.3	+2.6	20.5	0.86	1.03	+0.17	19.7	29.1	34.6	+5.5	18.9

The various fractions are expressed as per cent of the total electrophoretic pattern, as grams per 100 cc. of serum and as grams in the serum volume. Mean values from 11 men.

the first day of fasting and at the end of the fifth day just before food was taken.

Another group of six men was studied during a six-month period of semi-starvation and at intervals during 32 weeks of recovery. Three additional starvation subjects were studied during the first 12 weeks of recovery. The diet consumed during the period of semi-starvation was an European type of famine diet which averaged 1,654 Calories per day and contained 59 grams of protein; the general plan of the experiment has been presented elsewhere (9) and a few special aspects have been described (10, 11). The six months of restricted diet resulted in a 24 per cent loss of body weight and the development of the typical signs and symptoms of starvation, *i.e.*, fatigue, weakness, depression, polyuria, bradycardia and edema. During refeeding the weight of the men reported here was regained slowly. After six weeks only 15 per cent of the lost weight had been regained. Corresponding weight gains and time intervals were 12 weeks, 40 per cent; 20 weeks, 104 per cent; and 32 weeks, 107 per cent. Plasma volumes were determined on some of these men during both the semi-starvation and recovery period using the blue dye technique. The details have been presented elsewhere (10).

The electrophoresis was carried out in the Tiselius apparatus according to the technique described by Longsworth (12). A 90 mm. center section cell was employed to give good resolution of the pattern. Samples of sera were diluted with veronal buffer with an ionic strength and pH of 0.1 and 8.6, respectively. This mixture was dialyzed against the buffer in the cold (4° C.) for two days and then placed in the electrophoresis cell. The concentration of the serum protein at this time was between 2.5 and 3 grams per 100 ml. of solution. The patterns were analyzed by measuring the area under each peak with a planimeter after the area for each peak had been designated by the method of Tiselius and Kabat (13). The areas of the various components were measured on both the ascending and descending patterns and these were then averaged. Serum protein concentrations were determined by the micro-Kjeldahl procedure of Keys

(14) and the concentrations of the various electrophoretic components were calculated assuming a constant refractive index and nitrogen factor for all fractions, an assumption which does not lead to large errors in normal serum (15).

RESULTS

The effects of malaria

The effect of malaria on the distribution of the electrophoretic components of the serum proteins of the 11 subjects is summarized in Table I. It will be noted that there was no essential change in the concentration of total protein. There was

TABLE II

The comparison of the degree-hours of fever above 101° F. and the changes in terms of per cent of total pattern of the albumin, α_1 , beta and gamma fractions of the electrophoretic pattern

Subject Number	Degree Hours	Serum Protein Fractions			
		Alb.	Alpha 1	Beta	Gamma
19	147	-2.4	2.5	0.5	-0.3
18	164	-13.8	1.8	3.4	6.9
10	172	-13.6	3.0	3.1	8.2
16	179	-8.0	2.0	2.5	3.2
20	185	-8.5	3.0	1.5	1.9
9	193	-7.1	1.1	2.1	2.8
6	196	-8.5	3.3	1.5	2.7
7	197	-8.3	2.7	5.7	6.8
17	198	-4.7	2.9	3.6	-1.8
15	216	-8.0	3.7	1.8	2.8
11	221	-8.4	4.0	1.5	1.9

The subjects are arranged in order of increasing degree-hours.

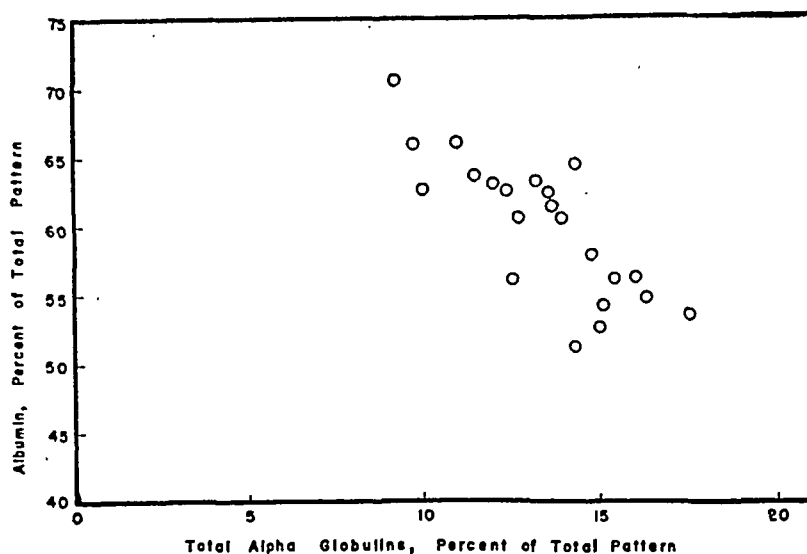


FIG. 1. THE RELATIONSHIP BETWEEN THE ALBUMIN EXPRESSED AS PER CENT OF TOTAL ELECTROPHORETIC PATTERN AND THE SUM OF THE α_1 AND α_2 GLOBULINS EXPRESSED IN THE SAME UNITS

Data are taken from the analysis of the sera of 11 men before and after a bout of malaria.

also no change in the circulating serum volumes of this group which averaged $3,383 \pm 287$ cc. before inoculation with *plasmodium vivax* and $3,365$ cc. ± 307 cc. after the last paroxysm. There was a 12 per cent reduction in the relative amount of albumin which represents an actual decrease in concentration and a loss of circulating albumin.

The relative areas of the α_1 and α_2 fractions were averaged and plotted against that of albumin. The results are presented in Figure 1. The product-moment correlation is 0.72, a figure

which is regarded by Snedecor as a significant correlation.

The extent of the changes in the electrophoretic pattern was compared with the number of degree-hours of fever above 101° F. The data are presented in Table II. The alpha globulin appears to be the only component which showed a change related to the number of degree-hours of fever above 101° F. The product-moment correlation is 0.54 between degree-hours and the percentage change of α_1 globulin.

TABLE III

The effect of $4\frac{1}{2}$ days of starvation and hard work on the electrophoretic diagrams of four men

Subject number	Total Proteins		Alb.		Alpha 1 Glob.		Alpha 2 Glob.		Beta Glob.		Gamma Glob.	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
11	6.99	7.70	63.0	67.8	3.7	3.2	5.7	4.4	12.0	10.6	15.6	14.0
6	7.02	8.81	64.0	67.0	3.3	2.7	6.2	5.0	11.0	9.5	15.5	15.8
18	6.90	7.62	63.8	66.2	4.6	3.8	7.7	6.4	10.1	9.3	13.8	14.3
7	6.68	7.30	65.5	68.5	3.9	3.4	8.0	6.6	13.6	12.6	9.0	8.9
Mean	6.90	7.86	64.1	67.4	3.9	3.3	6.9	5.6	11.7	10.5	13.4	13.2
Change		+0.96		+3.3		-0.6		-1.3		-1.2		-0.2

The protein concentration is expressed as grams per 100 cc. of serum and the relative amounts of the various electrophoretic fractions as per cent of the total pattern.

TABLE IV

The electrophoretic analysis of the sera of two men demonstrating the effect of 24 weeks of semi-starvation

Period	Subject No. 122						Subject No. 123					
	Control	S12	S24	R6	R12	R32	Control	S12	S24	R6	R12	R32
Total proteins gms./100 cc.	6.89	7.10	6.88	6.78	7.10	6.18	6.99	7.18	6.16	6.43	7.24	6.60
A/G	1.70	2.30	1.70	2.33	1.95	1.65	1.76	2.40	1.88	2.00	1.91	1.58
Per Cent of Total Pattern												
Alb.	62.8	69.6	63.0	70.0	66.2	62.2	63.7	70.6	65.4	66.7	65.5	61.2
Alpha 1	4.9	3.5	4.7	3.3	4.2	4.3	4.4	3.2	3.2	3.3	3.3	4.1
Alpha 2	8.4	7.7	9.7	7.1	8.0	8.4	7.6	6.4	7.5	7.0	7.2	9.2
Beta	11.8	10.5	12.9	11.6	13.0	15.2	11.2	9.9	13.7	13.2	13.1	15.0
Gamma	12.1	8.7	9.7	8.0	8.6	9.9	13.1	9.9	10.2	9.8	10.9	10.5

The analysis is presented for the control period, after 12 and 24 weeks of semi-starvation and after six, 12 and 32 weeks of rehabilitation.

The effects of acute starvation

The results of acute starvation are presented in Table III. There was a small but definite increase in the albumin fraction. This was accompanied by a decrease in both of the alpha globulins. The total protein concentration was increased by 14 per cent, a consequence presumably of the mild acidosis which accompanied the fast.

The effects of semi-starvation

Complete data are available on two men during the period of semi-starvation and recovery. This is presented in Table IV. There was an increase in the albumin fraction after 12 weeks of semi-starvation when these men had lost 18 per cent of their original body weight. The relative amount of albumin dropped somewhat after 24 weeks of semi-starvation when it was slightly above the value for the control period. During early rehabilitation it increased again slightly and then returned to normal by the end of 20 weeks when the original body weight had been recovered. There was a small decrease in the gamma globulin which was apparent at 12 weeks of semi-starvation and showed no further change after 24 weeks. It should be noted, however, that the gamma globulin had not reached control levels at the end of 32 weeks of recovery. The changes in the globulins of intermediate mobility do not appear to be significant except for the elevation of the beta fraction above the control levels at the end of recovery.

It had been planned to study six men throughout the course of the experiment but four samples of serum drawn at the end of semi-starvation were lost in an unfortunate refrigeration accident. The available data on these six men are presented in Table V. The results are similar and confirmatory to those found in Table IV.

Since the plasma volume tends to increase during starvation (10), the calculation of the total circulating protein clarifies the interpretation of the electrophoretic pattern. Plasma volumes were available for subjects 122 and 123 at the beginning and end of the semi-starvation pe-

TABLE V

Mean values from electrophoretic analyses of blood serum

Period	Total	A/G	Per Cent of Total Pattern					
			Alb.	Glob.	Alpha 1	Alpha 2	Beta	Gamma
Control	6.90	1.84	64.7	35.3	4.4	7.7	10.9	12.3
S12	7.15	2.31	69.5	30.5	3.3	6.8	10.2	10.2
R6	6.58	2.13	67.7	32.3	3.6	7.1	11.9	9.7
R12	7.14	2.02	66.6	33.4	3.5	7.3	12.2	10.4
R20	6.58	1.71	62.8	37.2	4.3	8.0	14.1	10.9
R34	6.47	1.62	61.6	38.4	4.1	8.2	14.9	11.2
30 Normals	7.01	1.72	63.0	37.0	4.1	8.0	12.0	12.9
Stand. Dev.	0.35	0.22	2.81		0.55	0.96	1.72	2.02

Results from the same six men are averaged for control, after 12 weeks of semi-starvation (S12) and after six and 12 weeks of rehabilitation (R6 and R12). Values for four men are averaged for the periods R20 and R34 (after 20 and 34 weeks of rehabilitation). For normal comparison, mean values and their standard deviations are given for 30 normal men. Values are in grams per 100 cc. for total serum proteins and as per cent of total proteins represented by the several fractions.

TABLE VI

The plasma volumes (in cubic centimeters) and the circulating fractions of the serum proteins (in grams) of two men before (C) and after 24 weeks of semi-starvation (S24)

Subject Number	Period	Plasma Vol.	Circulating Protein Fractions					Gamma
			Alb.	Glob.	Alpha 1	Alpha 2	Beta	
122	C	2746	118.9	70.3	9.3	15.9	22.4	22.8
	S24	3206	138.8	81.8	10.2	21.5	28.5	21.5
123	C	3059	136.1	77.7	9.5	16.2	23.8	28.1
	S24	3404	137.2	72.5	6.8	15.6	28.5	21.4
17 Normals		3249	140.7	79.1	9.4	17.2	24.9	27.7
Stand. Dev.		±305	±13.5	±10.0	±1.6	±2.5	±3.3	±5.6

For comparison the values of the same variable found in a group of 17 normals are presented along with their standard deviations.

riod. The circulating protein fractions in grams are presented in Table VI along with data for 17 normal young men. It will be noted that the only consistent changes were an increase in the beta globulin and a small decrease in the gamma fraction. The total circulating serum protein fractions for four men in the control period and after 12 weeks of semi-starvation are presented in Table VII. A definite increase in the albumin fraction and a small decrease in the circulating α_1 and gamma globulins are shown in this table.

Plasma volumes and electrophoretic analyses of sera were available on three additional men during the 12 weeks of recovery. These data are presented in Table VIII to illustrate the situation in early recovery. By R6 these men, who were in the group fed a "good" rehabilitation diet, had regained 20 per cent and at R12 they had recovered 53 per cent of the weight lost during semi-starvation. It will be noted that the decrease in the albumin fraction between R6 and R12 is due entirely to the data obtained from one man and

TABLE VII

The plasma volumes (in cubic centimeters) and the circulating fractions of the serum proteins (in grams) of four men before (C) and after 12 weeks of semi-starvation (S12)

Period	Subject No.	Plasma Volume	Circulating Protein Fractions					Gamma
			Alb.	Glob.	Alpha 1	Alpha 2	Beta	
C	123	3059	136.1	77.6	9.5	15.9	23.9	28.1
	119	2599	116.2	59.5	7.0	15.6	19.5	17.4
	129	3316	142.6	67.6	9.0	16.2	19.9	22.5
	26	3201	147.2	83.5	9.9	18.2	25.6	29.8
	mean.	3044	135.5	72.1	8.8	16.5	22.2	24.3
S12	123	2936	148.5	62.2	6.8	13.5	20.9	21.1
	119	2504	118.9	56.8	5.8	15.5	18.8	16.8
	129	3508	185.2	65.3	9.1	17.5	22.5	16.1
	26	3381	174.5	86.6	7.8	13.9	28.7	36.2
	mean.	3082	156.8	67.7	7.3	15.1	22.7	22.5

TABLE VIII

The plasma volume (in cubic centimeters) and the circulating fractions of the serum proteins (in grams) of three men after six weeks (R6) and 12 weeks (R12) of recovery from semi-starvation

Period	Subject No.	Plasma Volume	Circulating Protein Fractions					
			Alb.	Glob.	Alpha 1	Alpha 2	Beta	Gamma
R6	104	2972	126.6	64.8	6.2	18.4	23.2	17.0
	109	4182	173.9	98.3	10.0	21.3	33.9	33.0
	112	2695	121.8	77.1	7.0	18.3	26.9	24.8
	mean	3283	140.7	80.1	7.8	19.3	28.0	24.9
R12	104	2700	121.0	79.9	9.2	25.1	25.7	19.7
	109	3442	148.0	84.0	8.3	15.8	31.7	28.2
	112	2907	123.0	86.3	7.3	21.2	32.0	25.9
	mean	3016	131.0	83.3	8.3	20.7	29.8	24.6

For extent of recovery from the starved state see text.

that no change occurred in the total circulating gamma globulin.

DISCUSSION

The effects of malaria on the electrophoretic pattern confirm the negative correlation between the relative concentration of albumin and that of the alpha globulins reported by Chow (3). Malaria can no longer be regarded as an exception to the rule that acute infectious disease is accompanied by an increase in the alpha globulins.

It may be noted that when the total amounts of the various fractions in the circulation are calculated from the plasma volumes, albumin appears to be lost from the circulation and that there are definite additions to some of the globulin fractions. The effect of malaria on the electrophoretic pattern appears to differ from some other diseases such as scarlet fever (16) and rheumatic fever (16) in that no increase in the α_2 globulin accompanied the α_1 rise. Dole (17) has shown that attacks of relapsing malaria do not produce the increase in α_1 globulin reported here. The absence of this response in the malaria patient who has had several bouts with the disease might appear to be an adaptation to recurring stress.

Guttman *et al.* (18) studied the effects of malarial fever in syphilitic patients. These investigators used a phosphate buffer at pH 7.4 which does not separate α_1 globulin from the albumin fraction and this fact presumably accounts for

their failure to find any changes in the alpha globulins.

The decrease in albumin and the increase in alpha globulins which have been observed in many acute infectious diseases are not explicable as a result of a simple negative nitrogen balance such as is produced by a caloric deficit. The changes noted during malaria here are similar to those seen in pneumonia (19), scarlet fever (16), rheumatic fever (16), and tonsillitis (20). In contrast, no such changes were seen in acute starvation in spite of the fact that the subjects in the latter experiments lost roughly 50 grams of nitrogen each. This protein loss is comparable to that reported for some acute infectious diseases, including pneumonia and malaria (21, 22).

We have noted that, in our studies on malaria, there was little or no relation between the changes in albumin concentration and the number of hours of fever above 101° F. This is in contrast to reports, based on salting out with sodium sulfate, that the depression of the albumin concentration is related to the intensity of the malarial fever in both men (23) and monkeys (24). The difference may be related to the severity and duration of the disease; the malaria here was relatively mild and of short duration. However, our data indicate that the change in α_1 globulin is a more sensitive index to the severity of the disease than is the change in albumin content.

The results of the Minnesota semi-starvation

experiment clearly demonstrate that protein depletion due to simple caloric undernutrition does not explain the decreased relative albumin and increased alpha globulins shown in the electrophoretic patterns of sera from patients suffering from chronic debilitating diseases such as tuberculosis (25), ulcer (3), cancer (25) and rheumatoid arthritis (26). There can be no question that the subjects of the Minnesota starvation experiment were badly depleted of tissue protein. Corrections of the body weight loss (to be discussed elsewhere [27]) for changes in fat by the specific gravity method, for changes in extracellular water by the NASCN method and for changes in bone salt by X-ray density techniques indicate that the body weight loss underestimated the loss of active tissue, *i.e.*, more than 24 per cent of the original protein-containing tissues of the body were lost by these men.

It is apparent, then, that the alterations in the electrophoretic pattern of the serum proteins in the acute and chronic diseases mentioned above are not the results of protein depletion produced simply by a negative caloric balance. The question may be raised as to whether the "toxic destruction" of body protein can account for the picture in these diseases. The so-called catabolic phase (28) is defined empirically as a negative nitrogen balance which will not respond to an additional intake of calories or protein.

At the present time, the evidence is insufficient to decide whether the catabolic phase of protein metabolism is causally related to the characteristic alterations in the patterns seen in various diseases. Several facts suggest that the pattern changes are independent of this type of nitrogen loss. The negative nitrogen balance in tuberculosis, a disease which produces the alterations in pattern described above, is easily corrected by an increased intake of calories and protein (29). In addition, Peters (28) has presented evidence that the serum albumins, determined by the sodium sulfate technique, may increase in infectious diseases in spite of a markedly negative nitrogen balance. Finally, the same author states that after surgery the albumin concentration may decrease before the negative nitrogen balance becomes apparent.

The possibility cannot be ignored that the relatively decreased albumin and increased alpha

globulins observed in many diseases is a response to a certain type of injury and is independent of nitrogen loss over a wide range. It should be clearly understood that we are not denying that extreme protein depletion, *i.e.*, protein depletion resulting in more than 24 per cent weight loss, may result in decreased relative albumin concentration which may or may not be accompanied by a relative increase in the alpha globulin.

It is frequently stated that protein depletion results in a lowered resistance to infection (30). It is of interest, therefore, that the protein depletion produced in the Minnesota experiment resulted in only minimal reduction of circulating gamma globulins. While there is much evidence to indicate that the majority of circulating antibodies are found in the gamma globulin fraction (31), it has not been shown that gamma globulin is made up entirely of antibodies. Therefore, the small losses observed in semi-starvation in man do not necessarily represent depletion of circulating antibodies.

SUMMARY AND CONCLUSIONS

1. The sera of 12 normal young men were examined by the moving boundary method of electrophoresis before and after a bout of induced malaria. The malarial fever was allowed to run through five to eight paroxysms and an average of 193 hours over 101° F.

2. Changes in a given electrophoretic fraction after malaria were of the same order of magnitude regardless of whether they were expressed as per cent of total pattern, grams per 100 cc. of solution or as total circulating proteins. The albumin fraction decreased 14.5 per cent while the α_1 , beta and gamma globulins increased 62, 20 and 19 per cent, respectively.

3. The extent of the change in the α_1 globulin fraction after malaria more closely paralleled the number of degree-hours of fever over 101° F. than did the changes in any other fraction of the serum proteins.

4. Four men underwent acute starvation with hard work for five days which resulted in a 9 per cent loss of body weight. Electrophoretic analysis of the sera of these men before and after acute starvation demonstrated no important change other than a general increase in concentration due to a mild dehydration.

5. Nine subjects took part in a semi-starvation experiment which produced a 24 per cent loss of weight in the course of six months and the classical symptoms of famine, *i.e.*, weakness, fatigue, depression, bradycardia, polyuria and edema. The sera of two of these men were examined in the electrophoresis apparatus six times before and throughout both semi-starvation and recovery. The sera of the other seven men were examined at less frequent intervals. It was possible to calculate circulating plasma protein fractions for a number of these men.

6. The serum albumin fraction showed definite increases above normal after 12 weeks of semi-starvation and a loss of 18 per cent of the body weight. This was not accompanied by significant increases in other fractions. After 24 weeks of semi-starvation the albumin fraction had returned to normal. The albumin fraction was not depressed below normal at any time during the experiment.

7. Changes in the globulin fractions were minimal except for a definite increase in the beta fraction at the end of recovery when the men were a little overweight.

8. It is concluded that there are no changes in the electrophoretic diagram which are specifically characteristic of caloric undernutrition whether produced by complete starvation or semi-starvation.

9. At the present time, there is no valid evidence which relates the characteristic decrease in albumin and increase in the alpha globulins which are observed in many acute and chronic diseases to the protein deficiency of caloric undernutrition which may accompany these diseases.

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COMPLEMENT AND ITS COMPONENTS IN HUMAN CEREBROSPINAL FLUID¹

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It has been stated that normal human cerebrospinal fluid (CSF) does not contain complement (1, 2). Complement (C') has been noted, however, in CSF from occasional cases of meningitis and its presence has even been used as an indication of the permeability of the meningeo-vascular barrier (Weil & Kafka, in Katzenelbogen [1]). It has also been claimed that the absence of C' activity in normal CSF was due to lack of midpiece (3).

Since these observations were made, more sensitive methods for the estimation of C' have become available. These are based upon the spectrophotometric measurement (4) of the 50 per cent hemolytic unit (C'_{H50}) in the presence of adequate amounts of Ca^{++} and Mg^{++} (5, 6). Improvements have also been made in the tests for the four recognized components of C' (7). The question of the presence or absence of C' in spinal fluid was therefore reexamined with the help of these methods.

Because rather large quantities of CSF were required, samples collected prior to the taking of pneumo-encephalograms were used.² Cases showing infections of the central nervous system were excluded; otherwise, few of the samples could be considered entirely normal CSF. The fluids are listed in the table in order of increasing total protein content, with the hospital discharge diagnoses of the patients. All samples were examined for red blood cells, and when unusual numbers of these were found the fluids were excluded, since any C' found might have been due to adventitious admixture with blood.

About one-third of the fluids showed C' activity when tested directly by addition of 4 or 5 ml. to 1.5 ml. of a suspension of sensitized sheep red cells and sufficient buffer to give a total volume of 7.5 ml. as described by

Mayer *et al.* (4-6). Samples showing less than 3 per cent lysis are listed as non-lytic since the optical densities in the spectrophotometer were roughly only double those of the blanks.

At this low level, however, the test for C' is extremely insensitive as the von Krogh equation relating the quantity of C' to hemolysis (4, 5) indicates that 0.5 of a 50 per cent hemolytic unit is required to give 3 per cent hemolysis. An indirect method of estimation of C' activity was therefore considered more appropriate, and the samples were also tested by addition of preferably several different volumes to an accurately estimated portion of diluted human serum C' approximating a 50 per cent hemolytic unit, 1.5 ml. of a suspension of sensitized red cells and sufficient buffer as above.

The quantity of human C' to be used may be determined as follows: A 1:50, 1:75, or 1:100 dilution of human serum, depending upon its C' activity, is made up in the saline-veronal buffer containing 0.0005 M $MgCl_2$ and 0.00015 M $CaCl_2$ as described by Mayer *et al.* (5). With the aid of calibrated pipettes, accurately measured 1.5, 2.0, and 2.5 ml. portions of the diluted serum are mixed into 50 ml. centrifuge tubes containing 1.5 ml. (5×10^8) sensitized sheep erythrocytes and the above buffer, to make a final volume of 7.5 ml. The tubes are capped and, after incubation in a water bath at 37° C. for 45 min., with occasional shaking, are centrifuged for 10 min. at 2000 r.p.m. The supernatants are decanted with care and the degree of hemolysis is estimated spectrophotometrically and the C'_{H50} calculated (4). The optimal amount of human C' to which to add the CSF is in the range 0.8 to 0.9 C'_{H50} , and in this range an increase of 0.03 C'_{H50} should be significant. The human C' , if sterile, may best be preserved in small sealed glass ampoules in a CO_2 box. Once it has been standardized, a fresh ampoule may be opened for each set of CSF, but blanks, in duplicate, containing the predetermined volume of C' dilution and the hemolytic system should always be run along with the tubes containing C' and CSF.

In all but two CSF samples tested with a fraction of a C'_{H50} of human C' , an increase in hemolysis was noted over that of the C' itself. The results of these experiments are given in Table I as the difference in 50 per cent hemolytic units (C'_{H50}) above that of the control human C' dilution alone. These values are not all directly comparable, since only nine samples were tested at different volumes and six with more than one fraction of a C'_{H50} . For example, CSF 21 was tested by addition of 1.0, 2.0, and 4.0 ml. to 0.8 C'_{H50} of human C' and

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² The authors wish to thank Dr. Elvin A. Kabat, Neurological Institute, New York City, and Dr. Hattie E. Alexander, Babies' Hospital, New York City, for the fluids.

TABLE I

Complement and its components in spinal fluids from patients with various conditions

Laboratory number of cerebrospinal fluid	Diagnosis	Total protein* mg. per 100 ml.	Evidence of complement content		Tests for components of C'			
			Lysis by 4 ml.	Increase in 50% hemolytic units by 4 ml. + serum C' over lysis by serum C' control	C'1	C'2	C'3	C'4
9	Post-traumatic encephalopathy	7	0	0	±	+		
10	Low I.Q.	19	0	0.06				
12	Suspected brain tumor	22	0	0.10	++++, +++++	+, ±	+++±	+±
6	None	23	0†	0.14†				
1	None	24	4†	0.02†				
13	Idiopathic epilepsy	28	0	0.27, 0.13‡				
7	Diffuse cortical atrophy	29	3†	0.07†	+++	±		
15	Encephalopathy due to trauma	(30)	3	0.12	+++±	+, +	+++±, +++++	±, +++±
18	Mild cerebral atrophy	(32)	0	0.08, 0.09				
5	Post-concussion syndrome	33	5†	0.24†				
8	Suspected cerebellar tumor	35	0	0(?)	++++, +++±	+, +	+++	+±
26	Cerebral atrophy and arteriosclerosis	(36)	0	0.06				
19	Glioma	(38)	0	0.22				
28	None	40	0	0.11	+++±		+±	++++±
2	None	41	69†	0.50†	++++	+, ±		
16	None	41	0	0.07	+++	±, +	+++±	+++±
22	Cerebral atrophy	46	0, 0	0.10, 0.19§	++++	±, +	+++±	+++±
14	Arteriosclerotic encephalopathy	(50)	5, 2	0.46, 0.13				
20	Suspected brain tumor, diffuse cortical atrophy	(60)	0	0.39	++++	+, +	++++	++
11	Glioblastoma	84	0	0.16**				
21	Glioblastoma	92	0, 0	0.55††	++++	++	++++	++++
29	Brain tumor, type undetd.	100	22†	0.53††				

* Analysés by the laboratories of the Neurological Institute and the Babies' Hospital. Values in parentheses were carried out on another sample taken within one week.

† 5.0 ml. CSF used. 1.0 ml. of CSF 5 increased the activity of human C' by 0.1 C'_{H50}; 1.0 ml. of CSF 6 gave an increase of 0.03 C'_{H50}; 1.0 ml. of CSF 7 gave no measurable increase. The value of 0.02 C'_{H50} for CSF 1 is considered significant only in connection with the positive direct test.

‡ The second value was obtained after storage in the CO₂ box for 5.5 months in a rubber-capped bottle. The sample absorbed much CO₂ and the acidity was neutralized with 0.1 N and N NaOH before the tests were run. Identical values were obtained with 2.5 and with 4.0 ml. of the sample, indicating an anticomplementary effect for the larger amount. This occurred whether the additions were made to 0.6 or to 1.2 C'_{H50} of human C'.

§ After 4.5 months in the dry ice box.

|| After five months in the dry ice box.

¶ 3.0 ml. gave only 10% hemolysis, but this corresponds to 0.62 C'_{H50}, a value proportional to that for 4.0 ml., 0.78. Hence the sample had no anticomplementary effect in the larger amount.

** Although negative in its original state, a portion, dialyzed under negative pressure in the cold to one-fourth its volume in a period of five days, showed 7% lysis on the direct test with 4.0 ml. and 0.31 increase in C'_{H50} with 2.0 ml. on the indirect test, equivalent to 2% and 0.16 C'_{H50} on the original volume, with no allowance for deterioration. The initial indirect test showed 0.03 C'_{H50}.

†† With 0.84 C'_{H50} of human C', 1.0 ml. CSF 21 gave an increase of 0.15 C'_{H50}, 2.0 ml. an increase of 0.30. In another experiment with 1.27 C'_{H50}, 3.5 ml. gave an increase of only 0.20.

‡‡ Obtained with 3.0 ml. An increase of 0.33 C'_{H50} was given by 2.0 ml. This sample, also, was not anticomplementary in the quantities used. In this instance, also, there was little difference whether the test was carried out with 0.9 or with 1.3 C'_{H50}. It appears preferable, however, to add the CSF to somewhat less than 1 C'_{H50} of human C', since the lytic curve flattens out greatly above 1.3 units and the uncertainties of analysis are therefore greater.

showed a greater increase in lysis per milliliter of CSF the smaller the amount of CSF used. A 1.0 ml. portion of CSF 5 also showed a relatively greater increase in lysis than the 4.0 ml. sample, but this was not true of CSF 6. Similarly, CSF 13 caused a relatively greater increase at the 2.5 ml. level than at 4.0 ml., but CSF 14, 18, and 22 did not. It is therefore probable that the results given for some of the other CSF samples do not represent maximal values but reflect instead a balance between the activity of C' and the anticomplementary properties of the individual CSF.

Qualitative tests of C' components were also made in many instances, with reagents for C'1 and C'4 prepared from guinea pig serum according to Bier *et al.* (7). Tests for C'3 were made with a reagent obtained by incubation of 1.0 ml. of guinea pig serum with 0.4 ml. of a 1:1000 dilution of "Liquoid" in 0.85 per cent saline for 15 min. at 37° C. and dilution of the resulting mixture with saline to ten times the original serum volume.^a C'2 was tested for indirectly by noting any increased lysis of sensitized sheep red cells over that caused by a fraction of a unit of human C' in the presence of heat-inactivated guinea pig C' to provide adequate amounts of C'3 (*cf.* 7). The results are reported in the table as the highest degree of lysis from 0 to ++++ above that of the reagent controls. In tests for C'1, C'3, and C'4 the reagent controls showed no lysis but in two series of tests for C'2 the human C' plus inactivated guinea pig C' controls gave ++ and +++ hemolysis, respectively. In the table, therefore, + for C'2 represents an increase in lysis to +++ or ++++, respectively.

The tests for components indicated that all were present in varying amounts in human CSF without any obvious correlation between the strength of the test for a component and total protein content of the CSF. It is difficult to account for the failure of CSF 8, 12, 16, and 21 to show lysis in the direct test when all four components of C' could be shown to be present and when CSF 21, especially, gave such high values at different levels in the indirect test. Possibly, even though C'2, the limiting component of human complement (7), was present in these samples its amount may have been insufficient either to equal a measurable fraction of a C'_{H50} in 4 ml., the largest quantity used in the direct test, or to overcome the anticomplementary properties characteristic of this larger quantity and not present in 1 or 2 ml. Moreover, the tests for the components were carried out in a volume of 0.8 ml., so that the con-

ditions of these tests were very different from those for total hemolytic activity.

A surprising feature of the component tests was the weakness of many of those for C'4, a component usually present in very high titer in the C' of human serum (7). No attempt was made to titrate the level of C'4.

In some of the cases of this series, such as those with post-traumatic encephalopathies or with brain tumors with CSF in the high total protein range, the presence of C' might be accounted for by alterations in the permeability of the meningo-vascular barrier. There are, nevertheless, other CSF samples showing C' activity in which the total protein was within the normal range and the diagnosis indicated no disturbance which would be expected to alter the permeability of the barrier.

The principal outcome of the present studies, therefore, has been to demonstrate the presence of C' or its components in almost all of a series of 22 CSF samples, without obvious relation to the diagnoses of the cases from which the samples were taken or to the protein content of the fluids. Owing to the limitations of the tests themselves and the difficulties of their interpretation it would require study of a very large series of CSF samples before satisfactory answers could be given to questions relating the presence or absence of C' or its components in CSF to the physiology or pathology of the meningo-vascular barrier, or to the diagnosis of any particular disease. It is hoped that the positive findings, on the other hand, will serve to encourage a more detailed and lengthy study.

SUMMARY

1. About one-third of the samples of CSF taken prior to pneumo-encephalography showed hemolytic C' activity when tested directly with sensitized sheep red cells.
2. All but two of 22 samples tested increased lysis by a fractional unit of serum C'.
3. Qualitative tests for the four components of C' indicated their presence in those samples in which tests were made for all.
4. C' activity has been demonstrated in CSF containing protein in normal concentration and in CSF from patients with clinical diagnoses

^a Unpublished communication from Professor Graciela Leyton, Santiago, Chile.

compatible with a normal permeability of the meningo-vascular barrier.

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ASSOCIATION ANNOUNCEMENT

The 41st annual meeting of the American Society for Clinical Investigation will be held at the Chalfonte-Haddon Hall, Atlantic City, N. J., on Monday, May 2, 1949, at 9 a.m.

For those who may be interested, the annual meeting of the American Association for Research in Psychosomatic Problems will be held at the same hotel on Saturday, April 30, at 9 a.m., and Sunday morning.

The annual meeting of the Association of American Physicians will be held on Tuesday and Wednesday, May 3 and 4, also at the Chalfonte-Haddon Hall.

HEMATOLOGIC OBSERVATIONS IN PATIENTS WITH CHRONIC HEPATIC INSUFFICIENCY STERNAL BONE MARROW MORPHOLOGY AND BONE MARROW PLASMACYTOSIS^{1, 2}

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Macrocytic anemia is found in many persons with chronic hepatic disease. The incidence recorded by different observers had varied from 18% to 89%. Observations reported by Wintrobe (1) set the incidence of macrocytosis at 40.9% in 44 persons with cirrhosis and 62.5% in eight persons with cirrhosis and malignant disease of the liver. However, the pathogenesis of the anemia has not been clearly defined. In 1933 Wintrobe and Shumacker (2) and in 1936 Wintrobe (1) suggested that the anemia was due to the inability of a chronically and extensively diseased liver to store the antipernicious anemia factor. This opinion was supported by Goldhamer, Isaacs and Sturgis (3) who found that the antianemic substance was either reduced or absent in the livers of persons with hepatic cirrhosis and macrocytic anemia. However, in 1938 Schiff, Rich, and Simon (4) recovered an antipernicious anemia substance from the livers of cirrhotics with macrocytic anemia; they inferred that the defect was one of utilization rather than storage of this factor.

Poor hematologic response to liver extract has been the rule (1, 5), although an occasional patient with portal cirrhosis will respond as dramatically as a person with pernicious anemia in relapse (2); some cases are said to have responded to folic acid (6). In spite of these inconsistencies, the prevailing hypothesis still classes the macrocytic anemia of chronic liver disease with the erythrocyte maturation factor deficiency anemias. This is true even though the only report on bone marrow morphology

failed to demonstrate megaloblastic maturation arrest (7). The data presented in this paper stress the normoblastic rather than megaloblastic type of bone marrow morphology found in the majority of patients with cirrhosis and macrocytic anemia. Thus any type of erythrocyte maturation factor defect becomes an unlikely etiologic possibility in these patients.] Observations are also presented which correlate bone marrow plasmacytosis with hyperglobulinemia in persons with cirrhosis and suggest a causal relationship.

MATERIAL AND METHODS

The subjects chosen for study were 30 consecutive patients with a primary diagnosis of portal cirrhosis uncomplicated by recent hemorrhage. The diagnosis was made by history, physical examination, and by laboratory tests of liver function. Each of these tests was done at least two times on each patient and included the bromsulfalein retention, thymol turbidity, thymol flocculation,³ cephalin-cholesterol flocculation,³ quantitative serum bilirubin,³ alkaline phosphatase,³ and prothrombin time. The amount of urobilinogen in the stool³ was determined in 11 cases. The serum albumin/globulin ratios were determined using a modified biuret method (8) and the Coleman Junior spectrophotometer. In 23 patients liver biopsies were done and the diagnosis was confirmed histologically.

The hematologic characteristics of each subject's peripheral blood were determined on two or more occasions. Reticulocyte counts were performed daily and erythrocyte and hemoglobin determinations were made every second day while the therapeutic effects of various agents were being investigated. Red and white blood cell counts were performed with pipettes and counting chambers certified by the U. S. Bureau of Standards. Hematocrit determinations were made on oxalated venous blood (4 mgm. of potassium oxalate and 6 mgm. ammonium oxalate per 5 cc. blood), centrifuged for 30 minutes in a Wintrobe tube at 3,000 r.p.m. Hemoglobin

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³ These tests were performed by the Gastric Laboratory of the Cincinnati General Hospital and the authors wish to acknowledge the cooperation of Dr. Leon Schiff, director of the Gastric Laboratory.

was determined as oxyhemoglobin with the Coleman Junior spectrophotometer. Reticulocyte and platelet counts were made by the wet technique using Dameshek's method (9). The cytology of the bone marrow obtained by sternal aspiration was studied one or more times. Cover slip preparations for cytologic study of capillary blood and sternal marrow were stained by Wright-Giemsa stains.

The differential counts of the sternal marrow were done independently by each of the authors. Agreement was excellent in each instance, and the average of the two counts was taken as the representative value. In each case 500 non-erythroid nucleated cells were counted. The erythroid nucleated cells were expressed as numbers per 100 non-erythroid nucleated cells.

During the period of hospitalization each of the patients was placed on a "hepatic regime." This included a high protein, high carbohydrate, high vitamin diet supplemented with crystalline B-complex vitamins, protein hydrolysates, ferrous sulfate, and such lipotropic substances as choline and methionine. Several patients were given crude liver extract ("Intraheptol") intravenously. In addition each patient was given crude or refined liver extract intramuscularly or folic acid orally. The crude

liver extract was given in 5 cc. doses three times weekly and the refined liver in doses of 50 U.S.P. units three times each week. The folic acid was given in single oral doses of 100 mgm. daily. Patient No. 3 was kept on a vitamin B-poor diet and after ten days of observation with only an insignificant fall in erythrocytes was given one-half lb. of ground beef daily for three weeks. Thereafter this patient was treated in the manner described above.

RESULTS OF PERIPHERAL BLOOD STUDIES

Of the 30 persons studied, 24 were anemic, *i.e.*, they had less than 4,000,000 erythrocytes per cubic millimeter. Twenty of these had a macrocytic anemia (MCV of 96 cubic microns or above) and four had a normocytic anemia. The cells usually were normochromic. The remaining six patients were not anemic. Stained smears of the peripheral blood revealed moderate macrocytosis with minimal anisocytosis, poikilocytosis and polychromatophilia. Hypersegmented poly-

TABLE I
Initial examination of peripheral blood on 30 patients with portal cirrhosis

Case	Patient	Sex	Age	RBC (millions)	Hb. (gms.)	Reticu- lyocytes (%)	Platelets	Hematocrit	MCV cu. μ	MCH γ	MCHC (%)	WBC	Differential WBC				
													Segmented neutrophils	Lymphocytes	Monocytes	Eosinophiles	Basophiles
1	M. W.	F	36	1.71	6.6	7.7	212,040	19	111	38	34	10,450	76	15	9	0	0
2	C. G.	F	57	1.75	5.5	5.4	101,540	19	108	31	29	11,500	80	7	8	5	0
3	C. R. G.	F	29	1.82	8.3	6.0	650,000	25	137	45	32	11,400	73	15	9	2	1
4	R. P.	F	60	2.39	7.6	6.0	172,080	23	96	32	33	7,200	56	26	12	6	0
5	F. R.	F	34	2.82	10.2	3.4	327,100	33	113	35	31	4,400	80	12	4	2	2
6	G. K.	M	47	2.84	10.5	4.8	224,360	31	109	37	34	6,700	67	22	10	0	1
7	M. H.	F	43	2.87	8.6	3.8	201,480	25	90	30	34	7,200	70	15	8	6	1
8	E. M.	M	46	2.95	10.8	5.1	119,720	31	106	36	35	9,350	87	7	5	1	0
9	D. T.	F	17	2.95	8.0	6.1	259,600	25	84	27	32	11,280	90	3	4	2	1
10	C. N.	M	64	3.01	11.8	5.1	273,910	35	116	39	34	4,200	68	24	6	2	0
11	W. J.	F	40	3.04	10.8	3.8	346,560	34	108	35	32	5,750	73	18	8	0	1
12	F. H.	M	65	3.10	9.8	2.7	285,200	30	96	31	33	5,250	67	29	4	0	0
13	C. K.	M	55	3.10	9.8	4.5	328,600	32	103	32	31	9,100	86	6	8	0	0
14	T. R.	M	43	3.30	12.0	1.7	233,220	37	109	36	32	8,550	78	10	12	0	0
15	R. T.	M	37	3.40	11.3	1.6	373,680	39	112	33	29	6,450	87	8	5	0	0
16	E. N.	M	33	3.56	11.3	7.5	512,640	35	98	32	32	8,650	72	23	4	0	1
17	H. R.	M	58	3.59	14.0	4.6	247,710	41	114	39	34	19,000	93	4	3	0	0
18	E. M.	M	40	3.63	13.1	1.4	268,620	37	101	36	35	5,600	64	26	8	1	1
19	F. A.	F	68	3.67	12.6	5.1	242,000	39	108	34	32	11,200	76	17	4	0	3
20	E. P.	F	51	3.77	14.0	4.6	418,470	40	106	37	35	7,450	64	23	12	0	1
21	L. D.	M	46	3.81	11.3	6.7	262,890	34	89	30	33	4,500	76	13	11	0	0
22	A. S.	M	31	3.81	12.6	3.3	251,460	39	102	33	32	4,250	56	27	12	4	1
23	L. B.	M	57	3.88	12.6	2.5	492,760	39	100	32	32	6,100	54	34	8	3	1
24	J. F.	M	59	3.90	11.3	1.4	245,552	36	90	29	31	3,700	56	27	15	1	1
25	A. M.	F	32	4.02	13.3	4.5	249,240	40	99	33	33	9,200	79	11	4	2	4
26	C. R. K.	M	50	4.07	12.5	13.5	390,372	38	93	31	33	5,450	66	19	11	2	2
27	G. F.	M	70	4.07	13.3	2.7	305,250	40	98	33	33	5,200	58	32	10	0	0
28	C. K.	M	77	4.17	13.7	2.5	362,790	43	103	33	32	5,150	62	30	6	1	1
29	H. S.	M	47	4.17	14.0	2.7	487,890	41	98	34	34	6,000	66	28	5	1	0
30	W. R.	M	59	5.33	15.3	2.0	373,100	48	90	29	32	10,900	68	24	6	1	1

TABLE II
Differential bone marrow cell counts in 30 subjects with portal cirrhosis

Case	Patient	Polymorphonuclear leucocytes (%)	Metamyelocytes (%)	Myelocytes "C" (%)	Myelocytes "B" (%)	Myelocytes "A" (%)	Myeloblasts (%)	Lymphocytes (%)	Monocytes (%)	Eosinophiles (%)	Eosinophilic myelocytes (%)	Basophiles (%)	Plasmacytes (%)	Other cells (%)	Megaloblasts per 100 WBC	Early erythroblasts per 100 WBC	Late erythroblasts per 100 WBC	Normoblasts per 100 WBC	Number of nucleated RBC per 100 WBC
1	M. W.	40.0	14.0	6.5	3.5	1.0	0	15.0	0	0.5	0.5	0	19.0	0	0.5	1.5	7.0	41.0	50.0
2	C. G.	31.5	11.5	9.0	3.5	3.5	0.5	10.5	0.5	8.0	3.0	1.0	16.0	1.5	0	1.0	7.0	76.5	84.5
3	C. R. G.	38.5	30.0	10.5	2.5	1.5	0.5	9.5	0.5	3.5	0.5	0	2.0	0.5	1.0	3.5	12.0	30.0	46.5
4	R. P.	40.5	14.5	12.0	5.0	2.0	0	10.0	0.5	2.5	0.5	0.5	7.5	4.5	0	0.5	2.0	21.0	23.5
5	F. R.	45.0	12.0	9.0	7.0	1.5	0.5	10.5	0	3.5	0.5	0	7.0	3.5	2.0	3.0	8.0	36.0	49.0
6	G. K.	41.5	13.0	10.5	5.5	0.5	0.5	9.5	1.5	3.0	0.5	0	9.5	4.5	0	1.5	3.5	54.0	59.0
7	M. H.	38.5	21.5	10.5	3.0	2.5	0	5.0	0.5	0.5	2.0	0	14.0	2.0	0	1.0	4.0	40.0	45.0
8	E. M.	25.0	14.0	7.5	4.5	5.5	2.5	13.0	1.5	3.0	0.5	0.5	13.0	9.5	0	2.0	8.5	118.0	128.5
9	D. T.	43.0	16.0	8.0	8.0	1.0	0.5	12.0	0.5	1.0	1.0	1.0	7.0	1.0	0	5.0	15.0	75.0	95.0
10	C. N.	39.5	16.0	6.5	4.5	1.5	0	11.5	0	3.5	1.0	0	10.0	6.0	0	1.5	11.0	76.0	88.5
11	W. J.	28.5	30.0	6.5	2.5	1.0	1.5	7.5	1.0	0.5	3.5	1.0	13.5	3.0	0	1.5	6.5	34.5	42.5
12	F. H.	32.5	21.5	7.5	1.5	2.5	1.5	12.5	0	7.0	3.5	1.5	6.5	2.0	0	0.5	1.0	26.0	27.5
13	C. K.	39.5	19.0	9.5	2.5	1.5	0	5.0	0.5	4.0	2.0	1.0	12.0	3.5	0	1.0	5.5	32.5	39.0
14	T. R.	35.0	21.0	9.0	3.5	2.5	1.5	16.0	0	1.5	0	0	7.0	3.0	3.0	12.0	28.0	48.0	91.0
15	R. T.	52.0	14.5	7.5	2.5	1.0	1.0	6.0	0.5	4.5	1.0	0	3.5	6.0	0.5	3.0	10.0	64.5	78.0
16	E. N.	29.5	18.5	4.5	2.0	1.5	1.0	10.5	0.5	1.0	2.0	0	23.5	5.5	0.5	3.0	5.0	65.0	73.5
17	H. R.	29.5	12.5	13.0	7.5	1.0	0.5	4.0	0.5	5.5	0.5	0.5	20.0	5.0	0	2.5	10.5	56.5	69.5
18	E. M.	43.5	15.0	6.0	3.0	1.5	1.5	14.5	1.0	1.0	1.0	2.0	8.0	2.0	0	1.0	4.5	32.0	37.5
19	F. A.	38.0	11.5	4.0	6.5	2.0	1.5	13.0	2.0	6.0	2.0	1.0	9.5	3.0	0	2.0	3.0	29.0	34.0
20	E. P.	34.0	16.5	10.0	3.5	1.0	0	9.5	0	5.5	1.5	0	14.0	4.5	0	3.0	10.5	96.0	109.5
21	L. D.	49.5	15.5	3.5	2.0	2.5	0.5	5.5	0	7.5	3.5	0	9.0	1.0	0	1.0	4.0	52.5	57.5
22	A. S.	37.5	11.5	9.0	3.0	1.0	1.0	13.5	0.5	1.5	6.5	0.5	10.5	4.0	0.5	1.5	9.0	83.5	94.5
23	L. B.	43.5	11.0	6.5	1.0	3.5	0	10.5	0.5	6.0	2.5	1.0	11.0	3.0	0	0.5	2.0	41.0	43.5
24	J. F.	45.5	21.5	10.0	3.0	1.0	0	7.5	0.5	4.0	1.0	0.5	2.5	3.0	0	1.0	5.0	15.0	21.0
25	A. M.	36.5	13.0	6.0	1.5	3.0	3.0	19.0	1.0	2.0	0.5	0.5	8.0	6.0	0	1.0	7.0	76.5	84.5
26	C. R. K.	17.5	14.0	18.0	9.0	4.0	0.5	15.0	1.0	5.0	3.0	0.5	6.0	6.5	0.5	3.0	5.0	58.0	66.5
27	G. F.	34.0	30.0	7.0	2.0	0.5	0.5	14.0	0.5	3.0	1.0	0	6.5	1.0	0	1.5	3.5	54.0	59.0
28	C. K.	27.0	13.0	5.0	5.0	3.0	0	19.0	0	5.0	2.0	2.0	15.0	4.0	1.0	3.0	9.5	78.0	91.5
29	H. S.	48.5	13.5	3.5	1.5	3.0	0	8.0	0.5	4.5	1.5	0	8.5	7.0	0	1.0	2.5	66.0	69.5
30	W. R.	32.5	16.0	8.0	4.5	0	1.5	10.5	0.5	3.5	1.0	0	15.5	6.5	0.5	1.5	7.0	41.0	50.0

morphonuclear leucocytes were not observed. Reticulocytosis, usually ranging from 2% to 7.7% but in one instance as high as 13.5%, was found even though these patients did not show evidence of recent hemorrhage. The guaiac test for occult blood in the stool was faintly positive in several of these patients; but the degree of reticulocytosis was correlated with an increased bilirubinemia much more closely than with such minimal evidence of bleeding. The hematologic characteristics found in these 30 patients with portal cirrhosis are recorded in Table I.

RESULTS OF BONE MARROW STUDIES

The bone marrow appeared normally cellular in 21 subjects and moderately hypocellular in nine. The cytologic aspects and distribution of nucleated cells of the erythroid series usually were normal, although in 11 subjects there was mod-

erate hyperplasia at the normoblast level (see Table II). Such hyperplasia was found in the most anemic patients. In only three patients (Nos. 3, 5, and 14) was there a suggestion of the "megaloblastic maturation arrest" so characteristic of pernicious anemia and related macrocytic anemias. These patients were remarkable in that they were the only ones with signs of advanced multiple B-complex vitamin deficiencies, and each had a dietary history consistent with a prolonged deficiency of extrinsic factor. One of these patients (Case No. 5) had 57° free hydrochloric acid in the gastric secretions following histamine stimulation. The other two had none. Unfortunately there was no opportunity to repeat the test in the latter cases.

The granulocytic cells were normal; there were no bizarre metamyelocytes. The lymphocyte percentages were at the upper limits of normal.

There was, however, a striking and consistent increase in the number of plasmacytes. In the 30 patients the average was 10.5% with extremes of 2% to 23.5%. Normally, in our laboratory plasmacytes recorded as a percentage of nucleated nonerythroid cells range from 0 to 3.5% with an average of 1%. This plasmacyte hyperplasia was approximately proportional to the degree of hyperglobulinemia found in these patients. Table III and Figure 1 illustrate this relationship. In addition to the 30 patients with portal cirrhosis, we have studied the bone marrow of seven persons with biliary cirrhosis and seven with infectious hepatitis as revealed by liver biopsies. In

TABLE III

Correlation between serum globulin and plasmacytosis in the bone marrows of 30 patients with cirrhosis

No.	Pt.	Serum protein gms./100 cc.			Average globu- lin gms./ 100 cc.	Bone mar- row plasma- cytes per cent	Average plasma- cytes per cent
		Total	Albu- min	Globu- lin			
3	C. R. G.	6.2	4.0	2.2	2.60	2.0	4.1%
24	J. F.	5.7	3.5	2.2		2.5	
12	F. H.	5.7	2.9	2.8		6.5	
15	R. T.	6.0	3.1	2.9		3.5	
26	C. R. K.	5.7	2.8	2.9		6.0	
9	D. T.	5.7	2.6	3.1	3.22	7.0	8.0%
25	A. M.	7.5	4.3	3.2		8.0	
10	C. N.	5.2	2.0	3.2		10.0	
5	F. R.	6.1	2.8	3.3		7.0	
18	E. M.	6.8	3.5	3.3		8.0	
27	G. F.	5.7	2.0	3.4	3.46	6.5	9.1%
19	F. A.	5.6	2.2	3.4		9.5	
14	T. R.	6.9	3.5	3.4		7.0	
6	G. K.	6.4	2.8	3.5		9.5	
8	E. M.	5.3	1.7	3.6		13.0	
30	W. R.	5.1	1.5	3.6	3.82	15.5	12.0%
29	H. S.	6.3	2.5	3.8		8.5	
21	L. D.	5.8	2.0	3.8		9.0	
23	L. B.	7.7	3.9	3.8		11.0	
2	C. G.	6.1	2.0	4.1		16.0	
16	E. N.	6.7	2.6	4.1	4.28	23.5	13.7%
28	C. K.	6.0	1.8	4.2		15.0	
13	C. K.	6.6	2.3	4.3		12.0	
4	R. P.	6.7	2.3	4.4		7.5	
22	A. S.	7.6	3.2	4.4		10.5	
7	M. H.	7.2	2.7	4.5	4.72	14.0	16.1%
20	E. P.	6.7	2.0	4.7		14.0	
17	H. R.	6.2	1.5	4.7		20.0	
1	M. W.	6.4	1.6	4.8		19.0	
11	W. J.	9.1	4.2	4.9		13.5	

The cases are arranged in the order of increasing globulin values. Under these conditions there is a similar progressive increase in the percentage of plasma cells.

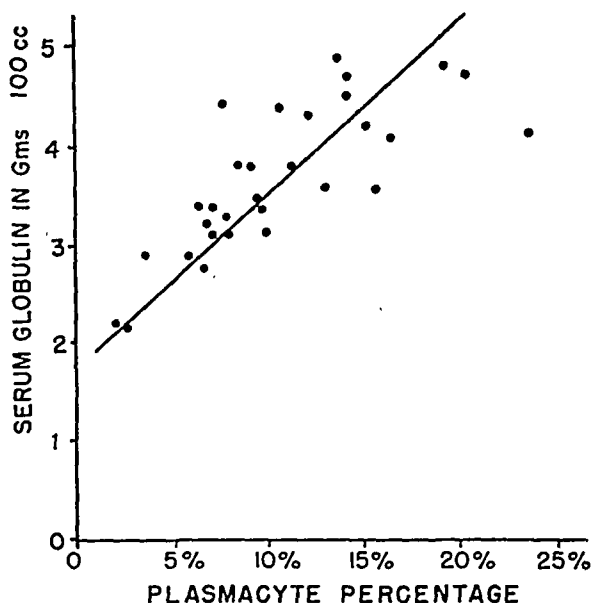


FIG. 1. GRAPHIC ILLUSTRATION OF DATA PRESENTED IN TABLE III

these 14 cases, unlike those with portal cirrhosis, plasmacytosis as high as 7% occurred only once and in none was there any correlation between the number of plasmacytes and the amount of serum globulin.

PROGRESS AND RESPONSE TO TREATMENT

There was no evidence of blood regeneration in the majority of patients on the hepatic regime supplemented with either liver extract or folic acid as previously described (see Figure 2). Slow non-specific hematologic improvement was observed in only five patients. This coincided with improvement in the patient's general condition.

A specific hematologic response similar to that which follows the administration of liver extract to persons with pernicious anemia was observed in only two patients (Nos. 3 and 14). A third patient (No. 5) who received one-half lb. of beef daily for 21 days in place of the hepatic regime, had an erythrocyte and hemoglobin rise of 2.75 to 3.72 millions and 10.3 to 12.2 grams respectively in this period of time. The reticulocytes did not increase during this period but were sustained between 4% and 5%, gradually falling toward the end of the period. The mean corpuscular volume fell from 120 to 108 cubic microns. Each of these three patients (Nos. 3, 5, and 14) had a significant number of megalocytes.

M.W., c.♀.36, PORTAL CIRRHOSIS, ALCOHOLIC

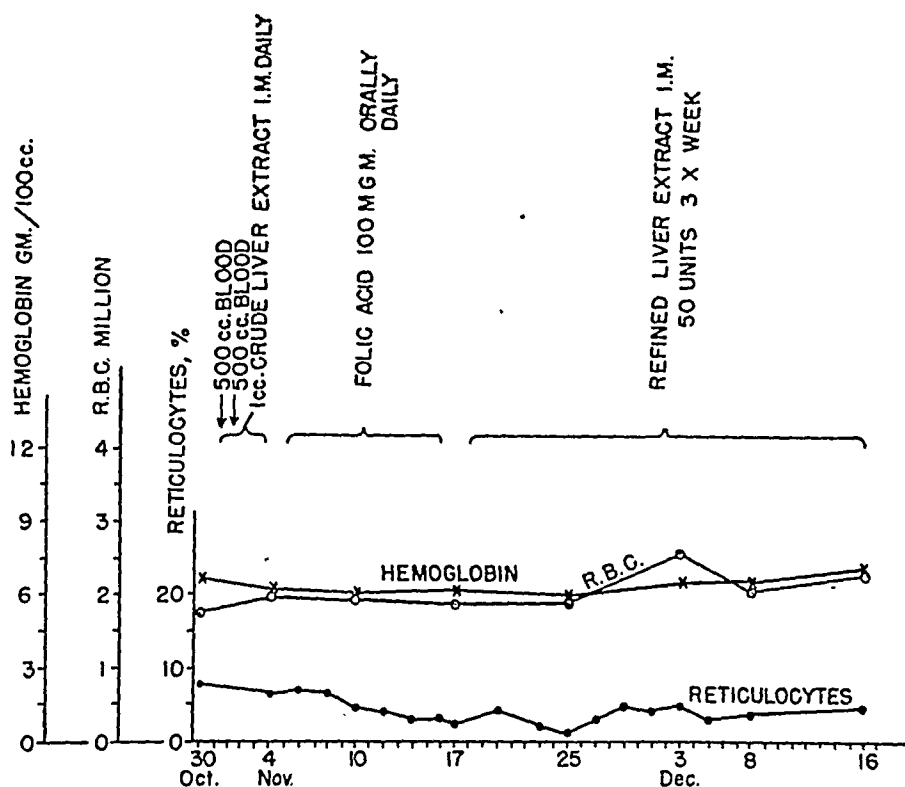


FIG. 2. THIS PATIENT HAD PROVED PORTAL CIRRHOSIS AND A MACROCYTIC ANEMIA

This graph illustrates the ineffectiveness of liver extract and folic acid in influencing the course of this anemia.

blasts and early erythroblasts in the bone marrow, but patients Nos. 3 and 14 were too ill to be subjected to the same experiment as patient No. 5. In fact patient No. 14 deteriorated rapidly even though there had been a hematologic response, and expired three weeks after hospital admission. Wintrobe (1) has recorded a similar experience.

DISCUSSION

The characteristics of the peripheral blood of the cirrhotic subjects in this study are comparable to those previously described (1, 5). Sixty-five per cent had macrocytic anemia; 15% had normocytic anemia and 20% no anemia. The accumulated evidence indicates that in the great majority of these subjects the macrocytic anemia of chronic liver disease occurred because of a metabolic defect other than erythrocyte maturation factor (liver factor) deficiency. The peripheral blood of persons with portal cirrhosis and macrocytic anemia did not reveal the anisocytosis, poikilocytosis, nucleated erythrocytes or multilobed poly-

morphonuclear leucocytes characteristic of pernicious anemia and related diseases. The bone marrow was normoblastic and either normally cellular or slightly hypocellular rather than hypercellular and megaloblastic. Finally, there was no change in the bone marrow or peripheral blood of persons with uncomplicated cirrhosis which could be credited to treatment with crude or refined liver extract or folic acid in large doses.

Three patients in this series had eaten diets for many years which were grossly deficient in B-complex vitamins, animal protein and presumably extrinsic factor. These were the only persons who exhibited advanced lesions of vitamin B-complex deficiency disease, such as glossitis, neuritis, and pellagrous dermatitis; these were the instances in which there were a significant number of megaloblasts or early erythroblasts in the bone marrow and in which there was a hematologic response to liver extract, and in one case, a response following the administration of ground beef. We believe that these three patients and the several similar patients reported in the medi-

cal literature (1, 10) are examples of extrinsic factor deficiency macrocytic anemia in persons with cirrhosis rather than the macrocytic anemia of chronic liver disease.

Several other points warrant brief comment. Blood loss cannot be invoked to explain the consistent initial reticulocytosis found in these patients and described previously by Rosenberg (5). Hemolysis or mild hypersplenism are unlikely causes because the level of urobilinogen in the stools was not elevated in any patient in whom the test was performed, and the bilirubin in the blood was usually of the direct reacting type. The uniform type of macrocytosis cannot be explained satisfactorily. Both of these problems require further investigation.

The bone marrow plasmacytosis in portal cirrhosis, which correlated roughly with the degree of hyperglobulinemia, has its counterpart in several other diseases and has been noted previously in European communications (11, 12). Such a correlation has also been found in lymphopathia venereum, Boeck's sarcoid, kala azar, multiple myeloma and serum sickness (13-17). As a result of studies on diseases associated with elevated serum globulin, Bing and Plum (18) conclude that the formation of globulin takes place in the plasmacyte. This opinion is supported by more recent investigations concerning the relationship of plasmacytosis to hyperimmune reactions and hyperglobulinemia (17-22). These studies indicate that plasmacytes develop in bone marrow and spleen in animals sensitized to bacterial or simple protein antigens as antibody globulin increases. Passively induced hyperglobulinemia or passive sensitization and shock do not induce a plasmacyte response.

Our studies do not indicate whether globulin is formed by plasmacytes or whether the plasmacytes appear in response to globulin formed elsewhere. Because of the studies cited above and by analogy with multiple myeloma it is likely that the plasmacyte is responsible for the excessive production of one of the beta or gamma globulins found by electrophoresis in the serum of patients with myeloma or portal cirrhosis (23). The stimulus to plasmacytosis in cirrhosis may be an antigen which the damaged liver either liberates or cannot inactivate.

Other globulins may be produced by lympho-

cytes and by the macrophages of the bone marrow, liver and other parts of the reticulo-endothelium without the mediation of the plasmacyte. Perhaps this is the reason why globulin may be elevated in acute hepatitis and biliary cirrhosis without a consistent increase in plasmacytes in bone marrow or other tissues.

CONCLUSIONS

1. A hematological survey has been carried out in 30 patients with proved portal cirrhosis.
2. Moderate or severe macrocytic anemia was present in 65% of the patients.
3. Reticulocytosis usually ranged up to 8% but in one instance was as high as 13.5%. It could not be explained by acute loss of blood.
4. The bone marrow of these persons was normally cellular or moderately hypocellular. In 11 there was moderate normoblastic hyperplasia. The only patients with megaloblastic maturation arrest had evidence of "extrinsic factor" deficiency.
5. Plasmacytes were consistently increased in the sternal bone marrows of these patients. The degree of plasmacytosis correlated roughly with the degree of hyperglobulinemia, suggesting a cause and effect relationship of a similar nature to that observed in multiple myeloma.
6. The macrocytic anemia failed to respond to liver extract and folic acid although there was an occasional spontaneous hematologic remission associated with a decrease in the severity of the cirrhosis. There was a significant hematologic response to liver extract or ground beef only in the three patients in whom extrinsic factor deficiency was suspected.
7. The normoblastic bone marrow and the lack of response to liver extract and folic acid in the majority of cases strongly suggests that the macrocytic anemia of chronic liver disease is produced by a metabolic defect entirely different from that responsible for pernicious and related macrocytic anemias.

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THE CARDIOVASCULAR EFFECTS OF ACUTELY INDUCED HYPOTHERMIA^{1,2}

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Under the conditions of relatively constant body temperature which exist in warm blooded animals including man, the physiological variations occurring in circulatory functions such as blood pressure, cardiac output, and circulatory rate are small. This relative constancy has led to the development of the concept of circulatory homeostasis. In recent years there has been a growing appreciation that the warm blooded animal may survive at greatly reduced body temperature during which marked circulatory changes may occur. The interdependence of the several cardiovascular functions has indicated the need for a correlation of the circulatory adjustments which occur during such progressive hypothermia. Only in this manner would it be possible to interpret the significance of the changes and their respective roles in the integration of the animal under the stress imposed by the hypothermia. Such studies may also have value in the development of rational therapy for individuals who have undergone prolonged exposure to cold.

METHOD

Fifteen heparinized dogs were anesthetized with intravenously administered sodium pentobarbital. Three animals were used as controls and 12 were cooled. Nine of these latter were maintained in a lighter and three in a deeper plane of anesthesia. The results were compared with those of six other dogs, in a parallel study (1), subjected to hyperthermia.

Respiratory rate and oxygen consumption were recorded with a basal metabolism apparatus attached to a tracheal cannula. Kymographic tracings of arterial blood pressure were obtained from the femoral artery

with a mercury manometer. Pulse rates were obtained from electrocardiograms taken simultaneously with the other measurements.

Cardiac output was calculated from the oxygen consumption and the arteriovenous oxygen difference according to Fick's formula (2). Two radio-opaque cardiac catheters were introduced through the external jugular veins and, with the aid of fluoroscopy, the distal ends were placed in the right auricle and in the pulmonary artery, respectively. The proximal ends of the catheters were connected with saline manometers for the recording of mean pressures. The location of the catheters and their zero pressures were verified at post-mortem examination. To ensure maximal mixing of the blood returning to the heart, samples of venous blood were taken from the pulmonary artery. Arterial blood was obtained from the cannulated femoral artery. In the course of each experiment, 100 to 150 cc. of blood were withdrawn and replaced with isotonic saline solution. The Van Slyke-Neill technic (3) was used in the analysis of blood O₂ and CO₂. Hematocrits were determined by the Wintrobe method (4), arterial blood being employed.

Control measurements were made when the blood pressure and respiration of the animal became stabilized. The animals were then covered with chipped-ice packs. Body temperatures were recorded from thermometers placed deeply in the rectosigmoid area and protected from the ice packs. The mean cooling period was about two hours during which the body temperatures fell to 29° C. The animal was then dried, covered with thin sheets, and exposed to radiant heat. However, the body temperatures continued to fall to 27° C, before beginning to rise. The mean rewarming period lasted five to six hours, at the end of which body temperatures usually had returned to 37° C. Ten or 11 determinations were obtained in each experiment, measurements being taken after each 2 degree change of body temperature.

Evaluation of the method

Measurements of right auricular and pulmonary arterial pressures represent only the approximate mean values. The changes in pressure were considered to be of greater significance. During periods of extreme bradypnea resulting from severe respiratory center depression, great variations in concentration of blood gases occur during each respiratory cycle, and this produces errors in the direct Fick method. This inaccuracy is further increased by the fact that the usual means of collecting blood samples makes it impossible to obtain exactly

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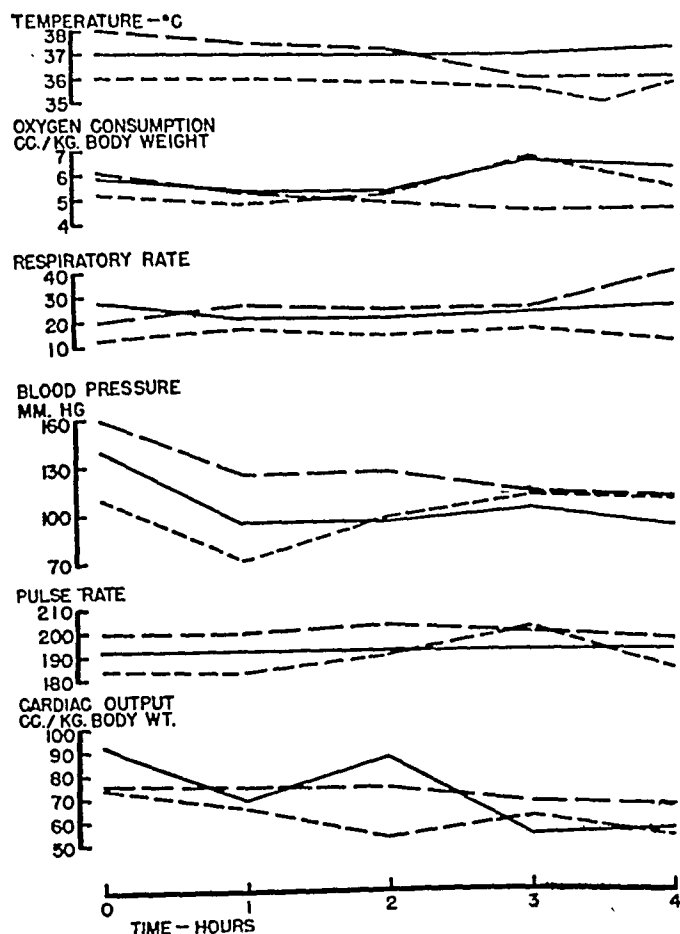


FIG. 1. COMPOSITE GRAPH OF DATA OBTAINED FROM THREE CONTROL DOGS, ANESTHETIZED BUT NOT SUBJECTED TO COOLING, DURING FOUR-HOUR PERIOD

Values for oxygen consumption and cardiac output are given in cc./kg./min. Respiratory and heart rates are in frequency per minute. The results in each individual dog are indicated by a different type of line.

simultaneously withdrawn arterial and mixed venous blood. These samples were drawn within 10 seconds of each other. This error is shown by the progressive accumulation of carbon dioxide in both arterial and venous bloods during apneic periods. Thus arterial blood taken at the end of apneic periods may contain more carbon dioxide than is found in venous blood withdrawn at the beginning of the respiratory cycle. For this reason, data obtained during marked bradypnea are not used in the conclusions of this study, although these are included in the tables.

Measurements similar to those described above were obtained over four-hour periods in a control group of three dogs, anesthetized but not subjected to cooling procedures (Figure 1).

The site from which true mixed venous blood can be obtained has been a controversial point, and has been discussed by many investigators in connection with the application of the direct Fick method in man. In the latter instance it has been demonstrated that there are only slight differences between blood taken from the right ventricle or pulmonary artery and that from the right auricle provided that the catheter tip is placed near

the tricuspid valve orifice. A different situation exists in dogs, our experience having shown that the oxygen content of blood taken from the right ventricle or pulmonary artery may differ markedly from that of the right auricle. We therefore adopted catheterization of the pulmonary artery as a more exact technic. Even greater errors may be introduced when venous blood is taken from the inferior vena cava due to the presence of more highly oxygenated renal venous blood which is poorly mixed with that from other viscera.

DISCUSSION OF RESULTS

Oxygen consumption

The initial values for oxygen consumption lay within a relatively small range, 4.0 to 6.6 cc./kg./min. In the control series no significant changes in oxygen consumption occurred during the four-hour period of observation (Figure 1). In eight cooled dogs there was a progressive decrease in oxygen consumption as body temperature fell. In two less deeply anesthetized animals the oxygen consumption increased from 4.7 to 10.8 and from 4.0 to 4.7 cc./kg./min. during shivering, and then suddenly decreased. In two other dogs the oxygen consumption did not vary significantly until body temperature fell to 31°C. Very little variation was noted in the pattern of change after the temperature fell below 31°C. The lowest oxygen consumption was 1.4 cc./kg./min. During rewarming periods there was a progressive increase in oxygen consumption in all instances. Data on three deeply anesthetized animals are given in Figure 2.

The direct relationship which was found between oxygen consumption and body temperature is similar to the results of other investigators in dogs (5). Variations in oxygen consumption up to 200% which we observed in the four less deeply anesthetized dogs mentioned above were associated with marked shivering and increase in pulse rate. There is general agreement that shivering causes an increase in oxygen consumption (6). Visible shivering occurred at temperatures as low as 29°C in our experiments and did not prevent further drop in body temperature. Figure 3 represents an experiment in which marked shivering did not prevent further fall in body temperature. It should be noted that the rate of temperature change during both the cooling and rewarming periods was not altered by marked shivering. The administration of 100% oxygen did not inhibit or delay the

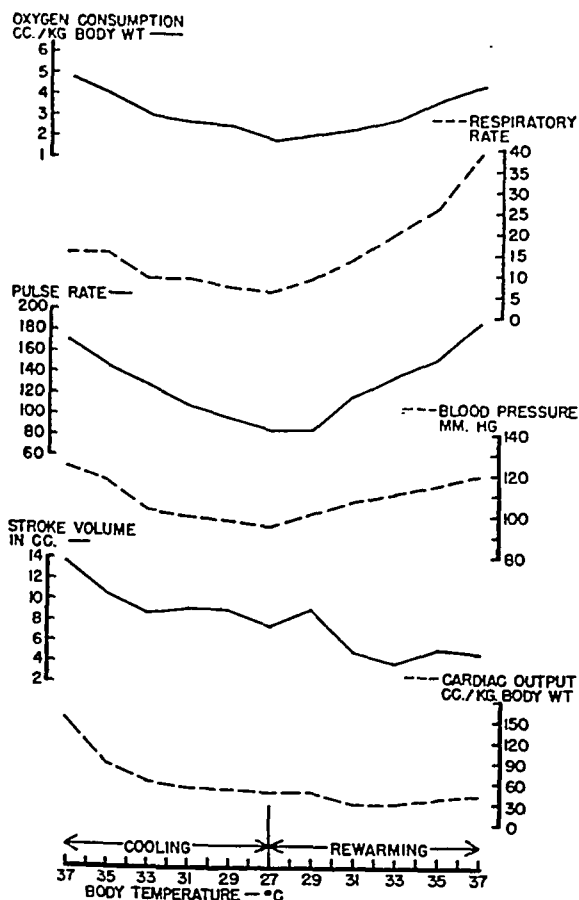


FIG. 2. A COMPOSITE GRAPH OF AVERAGE DATA OBTAINED FROM THREE DEEPLY ANESTHETIZED ANIMALS DURING COOLING AND REWARMING

It should be noted that the cardiac output failed to increase during the rewarming period despite the acceleration of the heart rate. Values for oxygen consumption and cardiac output are given in cc./kg./min., pulse rate is in beats per minute.

onset of shivering although such effects have been reported by others (7).

Respiratory rate

In the control series there was very little change in the respiratory rate (Figure 1). During both cooling and rewarming a close correlation between the respiratory rate and body temperature was found. This direct relationship was seen in the three deeply anesthetized (Figure 2) and in most of the less deeply anesthetized dogs. In two instances transient increases in respiratory rate from 28 to 40/min. and from 6 to 15/min. occurred during the early cooling period. The initial rates varied from 6 to 28/min., and at the

lowest temperature varied from 0.5 to 7/min. except in one dog. In this animal the lowest rate was 22, this dog maintaining a high rate during the entire experiment. In almost every case, the lowest respiratory rate occurred at the lowest body temperature. Following rewarming the range of variation in the rates was greater than that prior to cooling. Similar results have been obtained by others in animals (4, 8).

Pulse rate

The direct relationship found between changes in body temperature and pulse rate is shown in Figure 2. During the induction of cooling, increases in pulse rate occurred in the two animals

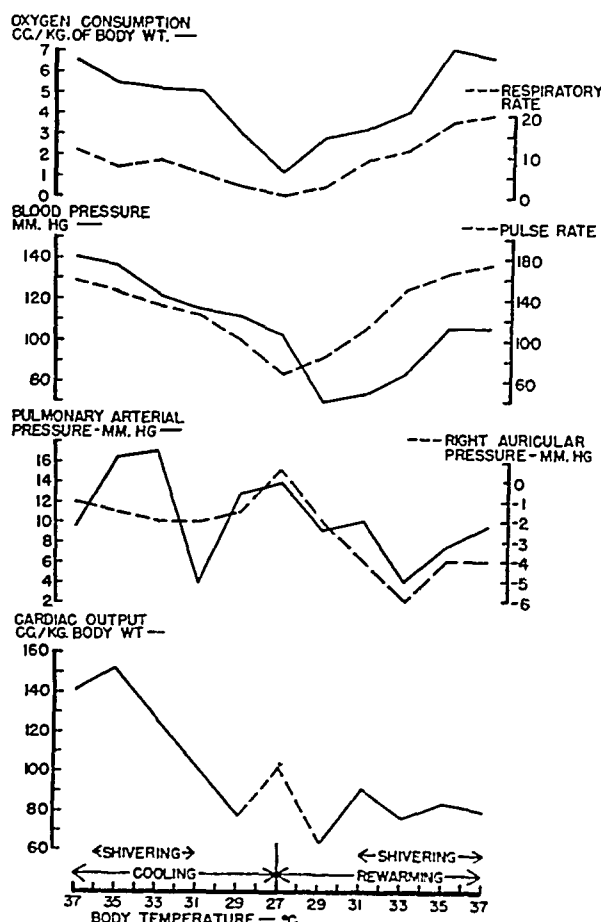


FIG. 3. A COMPOSITE GRAPH SHOWING THE EFFECT OF SHIVERING IN ONE DOG

It should be noted that the curves obtained in this animal do not differ significantly from those obtained from dogs in which shivering was absent (Figure 2). Values of oxygen consumption and cardiac output are given in cc./kg./min.

in which the rates were measured immediately after the ice was applied, although there was visible shivering in only one dog. In both there were transient increases in pulse and respiratory rates and blood pressure. After these initial changes an almost linear relationship was found, the changes in pulse rate closely paralleling the changes in body temperature. The lowest pulse rate was 59/min. occurring at 27° C. In the control series very slight changes were noted (Figure 1).

Similar results have been described by others (6). The direct effect of cold on the sinus pacemaker appears to be responsible for the variations in heart rate. The absence of changes in the control series lends support to the assumption that the changes are not due to the anesthetic.

Electrocardiogram

Electrocardiograms (Lead II) were taken simultaneously with other measurements in 11 dogs. The progressive sinus bradycardia occurring during cooling has already been discussed. There were no marked abnormalities of the P waves but the P-R intervals increased progressively as the heart rate slowed. A gradual widening of the ventricular complexes occurred during cooling, being maximal at the lowest temperatures in all but one dog. The average duration of the QRS complex was 0.062 seconds prior to cooling and this was doubled at 27° C. At the lowest temperatures an intraventricular block with notched S wave was noted in all but one animal. Figure 4 illustrates the relationship between the body temperature and QRS duration. Electrical systole, calculated from Bazett's formula, (9), was progressively increased from a normal value for K of 0.33 to 0.43 at 29° C (Figure 4). An elevation of the S-T segment was observed in nine dogs and a depression in one dog. In two dogs the T wave reversed its direction and in four there was a decreased amplitude. No change in T wave was noted in four dogs. Ventricular premature beats were seen in three dogs and in two instances, ventricular fibrillation developed.

The initial effect of cooling is on the heart rate and on the duration of electrical systole. Change in contour and duration of ventricular complexes and S-T-T changes occur later. The effect of cooling on repolarization in the ventricles is seen

before the effect on depolarization. All these changes were entirely reversible during the re-warming phase. The observed abnormalities are probably a direct effect of cold on the metabolic processes in the myocardium, since similar changes occur with direct application of cold (10). A more severe hypothermia than we used has been reported to induce auricular fibrillation and A-V block (11), but we did not observe these effects.

Blood pressure

Sodium pentobarbital anesthesia induces a fall in blood pressure, apparently via a direct effect on the vasomotor center and this is often associated with a spontaneous fall in body temperature (12). In confirmation of this we noted in the control series a fall in temperature of as much as 2° C, associated with a decrease in mean blood pressure during the first two hours of anesthesia; after this, the pressure was maintained at the lower level in two dogs and returned to control values in the third (Figure 1).

A slight but transient rise in blood pressure usually occurred immediately after the application of the ice packs. Shivering usually elicited further increases as great as 25%, but in two dogs, despite shivering, mean blood pressures fell 6% and 17% respectively. The fall in blood pressure which occurred during the early phase was

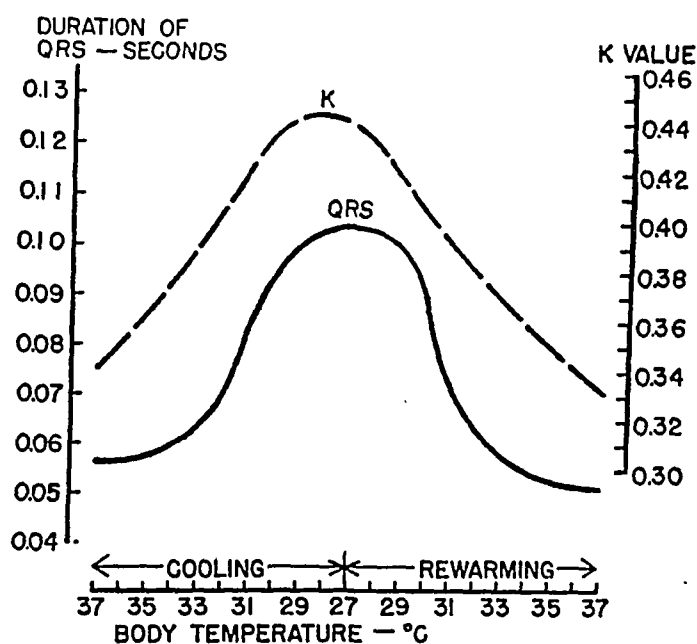


FIG. 4. RELATIONSHIP OF BODY TEMPERATURE TO ELECTROCARDIOGRAPHIC CHANGES

Discussed in text.

TABLE I
Cardiac output (cc./kg./min.) changes during cooling and rewarming

Dog no.	Cooling temperature ° C.								Warming temperature ° C.						
	38°	37°	35°	33°	31°	30°	29°	28°	27°	29°	31°	33°	35°	37°	38°
C2	150		70	59	56						36	34	46	49	
C7	175			43	47		39	30			30	29		46	
C11		144	100	83	66		63	35†		73	29	29	35	31	
C1	82	98*	111*	76*		129*		22			21	30	18†		
C3	72		96*		121*		53		46	77*	43	44	144†		
C5	63		143*	92			104†		80†	95*	68	64	57	66	66
C8		112	65	86	88		40		101†		78	67	82		
C9		75		39	173		42†								
C10	141		155	113*	168*		79		100†	63		72	86	80	
C12			200		77		†								

* Shivering.

† Dog died shortly afterward.

‡ Extreme bradypnea. Estimation inaccurate.

not great in most instances and not markedly different from the changes observed in the control series. However, during prolonged cooling, there was a tendency to a progressive fall in blood pressure and at the lowest body temperature in the dogs that survived, mean pressures had fallen to as low as 54% of the original level (Figure 2). During rewarming there was a consistent rise in blood pressure, the latter returning almost to initial values when the body temperature returned to 37° C.

This laboratory has previously called attention to a relationship between body temperature and blood pressure which may be demonstrated in several species of animals. In these, lowering of the temperature is accompanied by a fall in blood pressure (13, 14). Certain data in the literature on the effect of cooling on the blood pressure of man (15), and animals (16) are in accord with this concept. However, other data (17) show that the blood pressure may be maintained at control levels in cooled anesthetized rats until the body temperature falls below 29° C. Proskauer *et al.* (18) found that in rats an initial decrease was followed after 30 minutes of cooling by a rise in blood pressure. These responses may be a part of the homeostatic mechanisms which maintain the body temperature at normal levels, and in the face of a falling temperature maintain the blood pressure for a period.

Cardiac output

Control values for cardiac output in these and other dogs that we have studied averaged 112 cc./kg./min. and varied from 60 to 175 (Table I).

In the deeply anesthetized dogs there was a marked decrease in output during cooling and a small increase during rewarming (Figure 2). The lowest outputs occurred, not at the lowest body temperatures, but during the early rewarming periods, and these ranged between 35% and 56% of the control values. There were greater variations in cardiac output in the six less deeply anesthetized dogs. In 10 of the 12 dogs, there was a definite increase in output as great as 126% during the first period of cooling. These changes were associated with shivering, increased oxygen consumption and pulse rate, or a combination of these factors. For example, dog C1, which shivered violently during early cooling, showed an increase of oxygen consumption from 64 to 146 cc./min., of respiratory rate from 28 to 40 and of pulse rate from 176 to 196. The sudden rise in output in dog C9 at 31° C was related to an acute fall in arterial oxygen content which persisted until sudden vasomotor collapse led to death. This sudden increase in output may have been due to the acute anoxia but the etiology of the sudden drop in arterial oxygen content could not be accounted for. Complete recovery from cooling was not achieved as can be seen from the fact that the final values for cardiac output during rewarming were only about 50% of the original values. Increases in output during rewarming of the less deeply anesthetized dogs were more rapid and of a higher order and almost returned to the original values.

In two dogs of the control group there was a fall in cardiac output coincident with a spontaneous fall in body temperature up to 2° C (Figure 1). The reduction in cardiac output may have been due

partly to an anesthetic action and partly to the reduction of body temperature. No change in either body temperature or cardiac output occurred in the third control dog.

Right auricular and pulmonary arterial pressures

The mean right auricular pressure was measured in 10 dogs during cooling and in three controls, and was found to lie in the range of ± 1 mm. Hg in the control state (compared to the atmospheric pressure). In the control group variations occurred, usually in a negative direction, the largest being -2 mm. Hg. In the animals which were cooled the changes were as great as -5 mm. Hg.

The mean pulmonary arterial pressure was measured in eight cooled and three control dogs. The initial values lay in a range of 7 to 12 mm. Hg except in one dog in which it was 15 mm. Hg. In both control and cooled dogs there were rapid and substantial variations in pressures as much as 6 mm. Hg. The pressure changes were not consistent and bore no relationship to pulse rate, blood pressure, stroke volume or cardiac output.

With the development of the direct Fick method in man, much attention has been paid to the right auricular pressure and its relation to cardiac output. McMichael and his group (19) have promulgated the idea that within physiological limits in the compensated heart, the cardiac output changes concordantly with right auricular pressure but this concept has recently been criticized (20, 21). In our experiments there was no relationship between mean right auricular pressure and stroke volume, pulmonary arterial pressure, blood pressure or work of the right heart in either the cooled or the control series. Changes in the mean right auricular and mean pulmonary arterial pressures usually occurred in the same direction but were not proportional and on occasion the direction of change was discordant.

Hemoconcentration

Hematocrits were taken periodically in 10 dogs. It should be noted that about 100 to 150 cc. of blood were withdrawn and replaced with isotonic saline solution during each experiment. There was an average increase in the hematocrit value of 10% at the lowest temperatures with a tendency to hemodilution during rewarming. Barbour (22) has demonstrated a reflex which originates in

chilled skin and is mediated via the hypothalamus, to produce a shift in the body fluids from the circulating blood to the interstitial spaces in hypothermic states.

Causes of death

Five animals died in the course of these experiments. Cardiac failure has been suggested as a cause of death in hypothermia in man (23), in dogs (5) and in rats (17). However, this view is not substantiated by our observations. One death was due to ventricular fibrillation which occurred early in the cooling period; ventricular premature beats were frequent from the onset of the experiment and in this case an irritating effect of the intracardiac catheter could not be excluded. In three dogs there was a sudden fall of blood pressure prior to the cessation of respiration. This occurred during rewarming at body temperatures of 35° C in one dog, at 34° C, in another, and in the third dog it occurred at 27.5° C. Marked bradypnea and respiratory irregularity developed soon after the blood pressure fell and respiration ceased shortly thereafter. In these dogs the pulse rates were 146, 120 and 90/min. respectively, and the cardiac outputs were at high levels during the period immediately preceding the fall in blood pressure. We must conclude that in such cases there was a sudden vasomotor paralysis. In the fifth dog there was a primary central respiratory arrest. A sudden anoxic rise in blood pressure occurred and this was followed by a progressive fall in pressure. In this case the vasomotor center was able to respond to anoxia in a normal manner. Artificial respiration was instituted and the heart continued to beat for about two hours after respiratory arrest. Therefore, in our cases death was due to failure in the central nervous system either of the respiratory or of the vasomotor centers, as stated in some other reports (5, 24). In more prolonged and more intense hypothermia employed by others, other causes may be involved.

GENERAL COMMENTS

The present studies, correlated with those of other investigators, make possible a more complete analysis of the hemodynamic changes occurring during hypothermic states. Figure 2 represents a composite graph in which the relationship between the hemodynamic factors which we have

died, and changes in body temperature can easily be seen. The general effect of cold on all body tissues is to reduce progressively all cellular metabolic processes in accordance with van't Hoff's Law. Early in the cooling period of a warm blooded animal such as the dog, reflexes are inhibited which tend to decrease the loss of heat and to increase the rate of endogenous heat production, thus tending to maintain the body temperature at normal levels. Some of these reflexes result in a reduction in peripheral blood flow, shivering and redistribution of body fluids. The cardiovascular response during this early period is manifested by tachycardia, elevated blood pressure and increased cardiac output, as was observed early in the cooling of the lightly anesthetized animals. Anesthesia tends to inhibit these protective reflexes to some extent but shivering can be seen in most anesthetized animals during cooling.

As cooling becomes more intense and more prolonged there develops a progressive bradycardia, bradypnea, decreased oxygen consumption, decreased cardiac output and hypotension. The metabolic demands of the tissues for oxygen and substrate are so markedly decreased that true anoxia must be minimal despite a reduced availability of oxygen. This accounts for the failure of the administration of 100% oxygen to be of benefit or to prolong life during hypothermia. At very low body temperatures a progressive respiratory center depression occurs, due to the effect of cooling, and in our experiments to the effect of anesthesia. This depression may markedly reduce the oxygen supply and lead to the death of the animal in anoxia. During this phase of respiratory failure, oxygen and artificial respiration should be of value.

The depressant effect of cold on all body tissues holds true for the myocardium itself. This is reflected by the development of progressive sinus bradycardia, abnormalities in depolarization and later in repolarization, prolongation of electrical systole and the development of intraventricular block, as well as prolongation of auriculo-ventricular conduction time. The direct effect of cold on the heart is well shown by the development in the isolated heart preparation of changes similar to those described above. We observed no evidence of an inability of the heart to deal satisfac-

torily with the venous return of blood from the periphery.

The marked decrease of cardiac output which occurred during cooling is primarily the result of changes in the peripheral circulation. There is a progressive decrease in the circulating blood volume with the development of hemoconcentration, due to a shift of body fluids from the vascular bed into the interstitial spaces. These factors tend to reduce the venous return to the heart and to decrease the cardiac output. There are certain compensatory mechanisms, however, as is shown by the maintenance of the blood pressure at relatively high levels in the face of a markedly reduced cardiac output. These facts may thus be considered as evidence that marked peripheral vasoconstriction occurs, partially compensating for the decreased blood volume and cardiac output. When these compensatory mechanisms fail to meet the stress produced by hypothermia, vasomotor collapse occurs and leads to death.

All the changes which we observed were entirely reversible upon rewarming, and the recovery of function was in general related to the degree of rewarming accomplished. Thus the heart and respiratory rates, the blood pressure and the cardiac output tended to return to control values as the animal approached its normal body temperature. This would suggest that the induction of hypothermia in a warm blooded animal such as the dog may not be an altogether unphysiological procedure, in the sense that the compensating adjustment patterns, which appear during the cooling and the rewarming periods, are ordinarily capable of coping with the situation, within limits.

SUMMARY

1. Circulatory changes and cardiac output were studied in 12 dogs during cooling induced by exposure to chipped ice packs and during rewarming, and in three control dogs.
2. During the early cooling period, thermogenic reflexes which cause shivering are set off which act to produce an increase in oxygen consumption, respiratory and heart rates, blood pressure and cardiac output. These reflexes may be inhibited to some extent by deep anesthesia.
3. As cooling continues, and the combined pressing effects of hypothermia and anesthesia, hemoconcentration and a progressive fall in

gen consumption, in respiratory and heart rates, and in blood pressure occur. The cardiac output also falls. Marked prolongation of electrical systole, with the occurrence of intraventricular and A-V block, and changes in the S-T-T contour are seen. The changes in these values are related to the degree of hypothermia obtained.

4. During rewarming, these effects are reversed with a return of the blood pressure, respiratory rate, and pulse rate to normal, and with a tendency to a return to normal in the degree of hemocentration and cardiac output. Electrocardiographic changes were also reversed.

5. When it occurred, death in hypothermia was attributable to failure of the vasomotor or respiratory centers, rather than to failure of the heart.

6. No consistent correlated changes in right auricular pressure, on the one hand, and pulmonary arterial pressures, cardiac output, or the work of the right heart, on the other, were observed during either the cooling or rewarming periods.

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THE CIRCULATORY RESPONSES TO HYPERTHERMIA INDUCED BY RADIANT HEAT¹

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An elevation of the body temperature evokes many physiological changes, as a direct result of the increased rate of metabolism and the greater demand for oxygen and substrate, and indirectly in warm blooded animals because of the elicitation of thermolytic mechanisms. An understanding of the interrelationships between these factors is of obvious importance in an appreciation of the dynamics of hyperthermia and in the establishment of a rationale for its treatment. Despite many important contributions to this problem, there is a paucity of information concerning the effect of hyperthermia on the cardiac output and the right intracardiac pressures. We have therefore undertaken an analytical study of the circulation in hyperthermia in an effort to assay these adjustments more satisfactorily. Our present experiments have been limited to a study of the changes which occur following exposure to radiant heat since this method of producing hyperthermia seemed to be the simplest, being free from incidental effects produced by pyrogens and by other methods. A few experiments were done with short wave diathermy.

METHOD

Hyperthermia was induced in nine dogs. Six dogs, anesthetized with sodium pentobarbital, were exposed to radiant heat from several light bulbs until the body temperatures (about 38° C) gradually increased to 42° C during a two-hour interval. Body temperatures were reduced during the following one- to two-hour periods by using cold wet cloths applied to the body and by draft-producing electric fans. Temperatures were obtained from a thermometer placed deeply in the recto-sigmoid area. A mercury manometer attached to a

femoral artery recorded mean arterial blood pressures on a kymograph. Mean pulmonary arterial and right auricular pressures were obtained with saline manometers attached to their respective radio-opaque cardiac catheters. Cardiac output was calculated according to the Fick formula, blood from the pulmonary artery being used as mixed venous blood, and oxygen consumption being recorded with a basal metabolism apparatus attached to the cannulated trachea. Hematocrits of the arterial blood were determined according to the method of Wintrobe. Blood O₂ and CO₂ analyses were performed by the Van Slyke-Neill technique. A detailed description of these methods is given elsewhere (1). Measurements similar to the above were also obtained in a series of three dogs heated by short wave diathermy,⁵ but unless specified, the results given below refer only to those experiments in which radiant heat was employed. Similar measurements were made on three control anesthetized dogs kept at room temperature.

DISCUSSION OF RESULTS

Oxygen consumption

Many studies have demonstrated a close parallel between the rise in body temperature and the increased oxygen consumption (2-4) which accompanies the acceleration of cellular metabolism during fever. In our experiments (Figure 1) an average increase of 57% was observed at a body temperature of 42° C, the greatest increase being 100% in one instance. During the cooling period oxygen consumption decreased simultaneously with the fall in body temperature and returned to control levels as the temperature of the animal again reached 38° C. In the control series only slight and insignificant changes in oxygen consumption took place.

Respiration and content of blood gases

The production of hyperthermia was accompanied by an increase in respiratory rate and

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⁵ The Burdick Corporation generously supplied the short wave equipment.

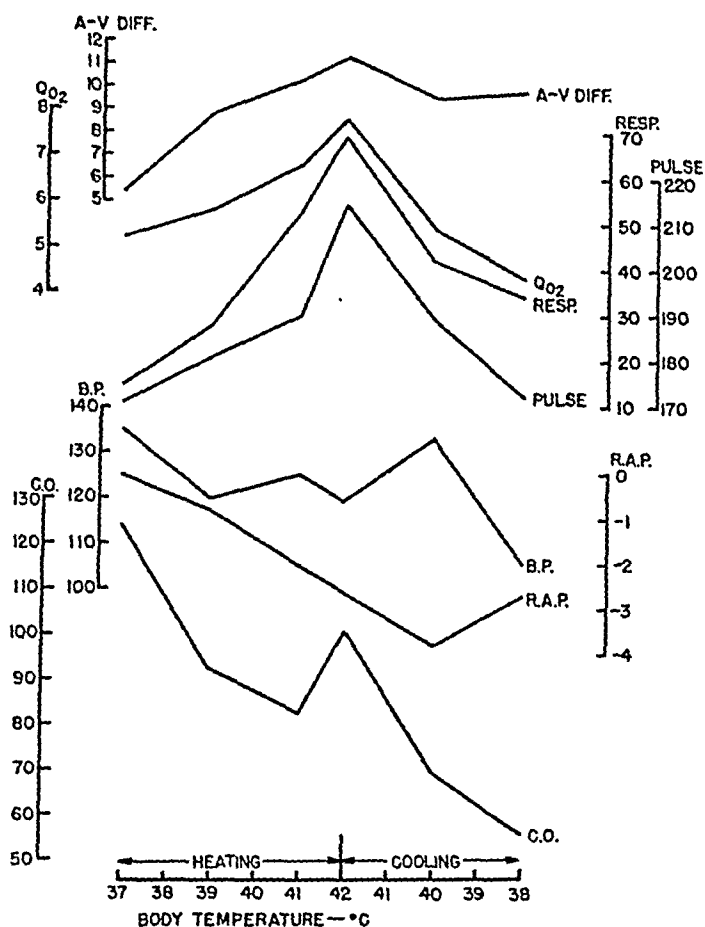


FIG. 1. A COMPOSITE GRAPH OF THE AVERAGE VALUES OF VARIOUS FACTORS DURING THE HEATING AND COOLING OF SIX DOGS

A-V Diff. represents the arteriovenous oxygen difference (vols.%); QO_2 , the oxygen consumption in cc./kg./min.; Resp. and Pulse are, respectively, the respiratory and pulse rates per minute; B.P., the mean arterial blood pressure in mm. Hg; C.O., the cardiac output in cc./kg./min.; and R.A.P., the mean right auricular pressure in cm. saline.

volume (Figure 1) similar to that which has been reported in all types of naturally occurring fever (2). During recooling the respiratory rate and volume decreased proportionately to the temperature fall. It is of interest to note, however, that greater than normal rates and volumes of respiration persisted even after the conclusion of the cooling period and the return of body temperature to control levels. This effect may have been due to the greater rate of destruction of the anesthetic agent during the febrile period and a consequent heightened activity of the respiratory center. In the control series no significant changes were noted.

Differences in the respiratory response were noted, depending on the type of heating used. With radiant heat the respirations were regular and only slightly decreased in depth. When the fever was produced with short wave diathermy, respirations became shallow and grossly irregular. The hyperpnea was probably due to the increased CO_2 production in the tissues, the increased activity of the respiratory center itself, and the initiation of thermolytic reflexes. It has been reported that extreme tachypnea and shallow respiration may seriously interfere with the exchange of alveolar air, and thus decrease the arterial oxygen content (3, 5). However, the slope of the oxygen consumption curve in our experiments indicated that the diffusion of oxygen in the lungs was not impaired when radiant heat was employed. With diathermy, the slope of the curve was highly irregular. It was because of this

TABLE I
Oxygen content of arterial and venous blood (volume per cent)

Body temperature	Dog no. H1		Dog no. H2		Dog no. H3		Dog no. H4		Dog no. H5		Dog no. H6	
	Art.	Ven.	Art.	Ven.	Art.	Ven.	Art.	Ven.	Art.	Ven.	Art.	Ven.
° C.												
36	—	—	12.6	6.3	—	—	24.1	20.8	—	—	—	—
37	20.6	13.6	—	—	—	—	25.1	16.2	—	—	—	—
38	—	—	11.6	3	12.8	7.5	—	—	—	—	14.9	12.1
39	19.7	10.4	11.7	2.4	13	5.5	23	8	15.5	11	16	12
41	19	8	12	2	12	2.7	24.5	9.6	19.4	6.3	16.2	11.4
42	19.7	5.7	12	1.9	12.1	—	25.3	10.1	—	—	16	10
40	19	8.7	—	—	13	5	—	—	20.3	7	16.4	8.7
39	—	—	—	—	—	—	24.8	6.3	—	—	—	—
38	18.9	8.7	—	—	12.7	6.0	—	—	20	4.6	15.4	7.9
37	—	—	—	—	—	—	24.6	3.2	—	—	—	—

Art. = arterial.
Ven. = venous.

irregularity that the results obtained with diathermy were not treated in the present study.

The arterial oxygen content varied only slightly during our experiments with an average decrease of 0.8 vol.%. The large difference in dog H5 (Table I) was associated with a simultaneous increase in hematocrit. No significant changes were noted in the control series. Others have reported decreases in arterial oxygen content during hyperpyrexia (3, 6) with a relatively greater decrease in venous blood oxygen content (3).

The effect of hyperpnea on the CO_2 content of the arterial and venous blood should be emphasized. Figure 2 shows that as the respiratory rate increased, the CO_2 content of the arterial blood decreased. The curve for venous blood is similar to that of arterial blood but shifted to the right. The primacy of the thermolytic mechanism which is effected chiefly by panting in the dog is seen by the marked reduction of blood CO_2 and the consequent alkalotic tendency. Despite this tendency to alkalosis, the respiratory rate continued at high levels.

Arteriovenous oxygen difference

The oxygen content of the arterial and venous bloods is given in Table I. It can be seen from

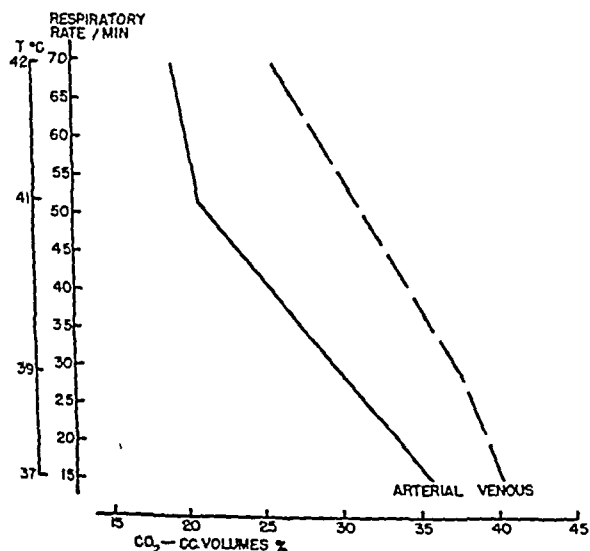


FIG. 2. A GRAPH SHOWING THE RELATIONSHIP BETWEEN THE BODY TEMPERATURE, RESPIRATORY RATE, AND THE CARBON DIOXIDE CONTENT (VOLS.%) OF ARTERIAL AND VENOUS BLOOD DURING THE WARMING PERIOD FROM 37° TO 42° C

T represents temperature.

it that the utilization of oxygen increased proportionately to the rise in body temperature despite the progressively reduced cardiac output (Figure 1). The average arteriovenous oxygen difference was 6.1 vol.% at 36° to 38° C and increased to 11.3 vol.% at 42° C, remaining high in some instances or even increasing during the re-cooling period. The increase in A-V oxygen difference was relatively greater than the increase in oxygen consumption. These findings are in general agreement with those of Uyeno (3).

Hematocrit

Approximately 100 cc. of blood were removed and replaced by isotonic saline solution during each experiment. Hematocrits of arterial blood samples showed no consistent trends. During fever it has been reported (7) that there is usually an initial hemodilution with increased blood volume.

Cardiac output

Two of the most important factors which must be assayed in order to understand the circulatory changes which occur during exposure to radiant heat include the simultaneous measurement of the cardiac output and the blood pressure. In our experiments the cardiac output decreased consistently with fever (Table II) in all but two animals in which a transient rise occurred at the beginning of the warming period. The output began to decrease early or immediately after the heat was applied. In the two dogs H2 and H4, the cardiac output decreased markedly while the temperature was rising from 36° to 38° C (from 1396 to 821 cc., and from 1300 to 712 cc./min., respectively). The relatively greater increase in the arteriovenous oxygen difference compared to

TABLE II
Cardiac output cc./kg./min. and body temperature

Dog no.	Body temperature (° C.)									
	36	37	38	39	41	42	40	39	38	37
H1	—	78	—	95	77	64	54	—	53	—
H2	89	52	64	—	—	57	died at 43° C.	—	—	—
H3	—	—	136	82	86	—	85	—	77	—
H4	111	64	—	41	49	50	—	20	—	12
H5	—	—	—	74	33	—	29	—	16	—
H6	—	—	344	166	163	129	81	—	75	—

that of the oxygen consumption accounted for the decreased cardiac output which occurred in the face of increases in oxygen consumption and pulse rate.

Electrocardiographic studies (2, 8) and the transitory character of the changes during fever suggest that the conduction system of the heart is apparently not damaged in short-lasting fever. We may therefore assume that the changes in cardiac output during fever were determined primarily by a decreased venous return rather than by cardiogenic factors. The decreased venous return is probably an early result of redistribution of blood which may later be accentuated by the markedly reduced blood volume resulting from the fluid loss consequent to heating. Some decrease in cardiac output was noted in the controls due partly to the anesthetic and partly, perhaps, to the spontaneous fall in body temperature of one to two degrees (1).

During recooling the cardiac output continued to decrease significantly, even after body temperature had fallen to control levels (Table II). This would suggest that the febrile period sets up mechanisms which reduce the cardiac output, and that these mechanisms may persist for a time after the cessation of the heating period.

Blood pressure

Blood pressure changes seen during the induced fever were variable (Figure 1). In four dogs an initial and progressive fall occurred. In one, no change in pressure occurred and in one there was an initial rise followed by a fall. The greatest fall in pressure was 57% of the original level. In two of the three dogs heated with short wave diathermy the blood pressure tended to rise and then remained at the higher levels, findings similar to those reported by Wiggers and Orias (9).

Since the blood pressure is the resultant of a number of factors, the effect of heating on each of them must be considered in the interpretation of results. Among the more important are the decreased cardiac output, an increase in the vascular bed due to vasodilation in various portions of the body, and neurogenic factors which tend to increase the peripheral resistance and thus maintain the blood pressure. The apparent contradiction in the latter two factors may be resolved on the

basis of regional differences in vascular tone. This mechanism deserves further analysis. In the control series, a slight decrease in blood pressure occurred probably due to the anesthetic and to the spontaneous fall in body temperature (1).

Pulse rate

A close correlation between body temperature and pulse rate has been shown (2). Acceleration of the pulse rate is effected by the direct action of the increased temperature on the sinus node which remains the pacemaker even during very rapid rates (10). The rate increases as much as 92 beats per minute at 42° C, averaging 39 (Figure 1). No significant changes were noted in the control series. An acceleration of the pulse rate is obviously not necessarily associated with an increase in cardiac output since other factors play more dominant roles (11).

Intracardiac pressures

The pressure in the right auricle was measured in four dogs and showed steady and substantial decreases (Figure 1). The pressures continued to fall during the cooling period and at the end of the experiments were as low as 4 cm. saline below the control levels. Large variations in both directions were seen in the pulmonary arterial pressure in both the heated and control series. The changes in the right auricular pressures in the control group were inconstant, varying only slightly from control levels.

DISCUSSION

The data collected during our experiments represent an analysis of the circulatory responses to exposure to an intense radiant heat. The mechanism of the rise in body temperature induced by exposure to radiant heat is essentially that of a prevention of heat loss (12). There is no doubt that adaptation will be different following exposure to different types of heat.

The generalized application of intense radiant heat was followed shortly by a progressive fall in cardiac output, regardless of the elevated body temperature, oxygen consumption and the heart rate. It has too often been assumed that cardiac output invariably increases during fever (2, 10). While the output may increase in certain circumstances (3, 4, 6) recent ballistocardiographic stud-

ies have failed to demonstrate an increase during fever (13) and others have suggested that the output may be reduced in hyperthermic states (9, 14). Our results demonstrate that in induced fever due to radiant heat, the cardiac output is usually diminished and that this is a dominant factor in the ensuing circulatory changes.

In hyperthermia produced by high external temperature, marked vasodilatation occurs in the cutaneous and other vascular beds (2). Large amounts of blood may therefore be shifted to the periphery, resulting in a decreased volume of blood available for venous return to the heart. This is indicated by the falling venous pressure which we observed in our hyperthermic animals. The increased respiratory activity associated with an excessive internal temperature and the loss of body fluids both contribute to decrease further the effective blood volume. These changes also lead to a reduction in venous return and so to a further reduction in cardiac output, in proportion to the duration and intensity of exposure to radiant heat.

Many explanations have been advanced to explain the circulatory collapse in heat stroke and these have been reviewed recently by Daily and Harrison (4). These include hemoconcentration, reduction of blood volume, increased intracapillary pressure resulting in increased fluid transudation and cardiogenic vascular collapse. Wiggers and Orias (9) have demonstrated a reduction in systolic discharge and a decrease in the force of ventricular contraction when the body temperature rises above 41° C due to exposure to radiant heat. Our results suggest that the normal heart is not significantly injured by hyperpyrexia and appears to be able to cope adequately with the venous return from the periphery.

Previous experiments in this laboratory (1) have re-emphasized that both man and animals may undergo prolonged exposure to cold with reduction in body temperature as great as 12° C in the dog, with complete recovery in most instances following rewarming. Tolerance to elevated body temperature is therefore considerably less than tolerance to cooling. Thus, in the dog, survival after a rise in temperature of 5° C will be rare and even lesser elevations of body temperature may be sufficient to embarrass the cardiovascular system.

When the febrile animal was permitted to lose heat, the body temperature rapidly returned to normal but the cardiac output continued to decrease. Evidently mechanisms initiated during the hyperthermic phase which reduced the cardiac output persisted during the phase of return to normal body temperature. Rational therapy should therefore be directed to measures which would increase the cardiac output to normal values during the cooling phase. Simple reduction of the body temperature to normal obviously would be inadequate since the cardiac output remains markedly reduced and peripheral circulatory failure may result in death.

SUMMARY

The changes in the cardiovascular system were studied in anesthetized dogs during acute hyperthermia induced by intense radiant heat and during the return of body temperature from 42° C to normal.

During the warming period the oxygen consumption increased markedly, rising to about 150% of normal at 42° C. The cardiac output was progressively reduced. As a consequence the arteriovenous oxygen difference was increased. The blood pressure was maintained at relatively normal levels in the face of an apparent increase in the capacity of the vascular bed and a coincident decreased cardiac output, indicating an active vasoconstriction process. The heart rate increased progressively with hyperthermia and the right auricular pressure tended to fall.

During the recooling period the oxygen consumption, respiratory rate and heart rate returned toward normal levels. The cardiac output and right auricular pressure continued to decline significantly even after the body temperature had returned to control levels. The blood pressure was maintained in most of the animals. No significant changes were found in the pulmonary arterial pressure.

During hyperthermia the heart appears to be able to cope with the venous return. Our experiments therefore suggest that the circulatory changes occurring in acute hyperthermia depend upon (1) an increased demand for blood by the tissues which leads to (2) an increase in the volume of the vascular bed. Since (3) the circulating blood volume and venous return do not in-

crease proportionately and may even decrease, a marked reduction in cardiac output occurs. (4) If the blood pressure and the circulation are to be maintained vasoconstriction must occur. Failure of the vasomotor centers to compensate for the progressively reduced cardiac output prepares the ground for peripheral vascular collapse.

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THE RELATION OF INFLUENZA VIRUS AND BACTERIA IN THE ETIOLOGY OF PNEUMONIA^{1, 2}

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Evidence is accumulating that influenza virus plays a role in the etiology of certain pneumonias generally considered to be primarily bacterial in origin. The earliest recorded observations (1, 2) describe influenza as a mild self-limited disease of the upper respiratory tract which progressed occasionally in certain individuals to produce more serious symptoms involving the pulmonary tissue. Soon after the discovery of bacteria, clinicians (3) pointed out that pneumonia caused by streptococci, staphylococci and pneumococci often followed symptoms of influenza.

During the pandemic of 1918 influenza was frequently complicated by bacterial pneumonia. Certain investigators (4, 5) believed that the primary disease was of unknown virus etiology and that the pulmonary involvement was a manifestation of the secondary invasion of the respiratory tissues by pathogenic organisms.

Epidemiological data (6-10) show a significant increase in pneumonia deaths accompanying and following epidemics of clinical influenza. Other similar data (11, 12) indicate that localized epidemics of pneumococcus pneumonia have been associated with outbreaks of clinical influenza.

Laboratory evidence (13-22) on clinical cases of bacterial pneumonia in man and experiments on laboratory animals (23-28) during the past decade have further supported these observations.

The studies reported herein show that there was a simultaneous infection with pneumococci and influenza virus in about one-half of the human cases of lobar pneumonia studied during an influenza epidemic. Such mixed infections were also observed during interepidemic periods.

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MATERIAL AND METHODS

Source and preservation of specimens

Sputum: Specimens of sputum were obtained from 69 cases of bacterial pneumonia admitted to hospitals in the Baltimore area between November 30, 1946 and August 5, 1947. The group included 61 cases of pneumococcal pneumonia, three cases of pneumonia due to Friedlander's bacillus, and five cases which were diagnosed clinically as bacterial pneumonia but in which the causative organism was not identified. Sixty-four were clinically lobar pneumonia and five were broncho-pneumonia. Sixty-two of the specimens were from the Osler Medical Clinic of The Johns Hopkins Hospital. These cases are believed to represent an unselected sample of the 75 cases of bacterial pneumonia which hospital records show were admitted to the Osler Clinic during this period. The other seven specimens were obtained from either Sinai Hospital or from the Veteran's Hospital, Fort Howard, Maryland.

Thirty-six of the 69 cases (52 per cent) occurred during March and April 1947, when influenza "A" was known to be prevalent in the community as indicated by an increase in upper respiratory disease and by the frequent isolation of influenza virus. The occurrence of this outbreak afforded the opportunity to classify the cases into two groups (1) interepidemic, those occurring before and after March and April 1947, and (2) epidemic, those occurring during March and April. This classification with respect to the influenza epidemic is used throughout this report.

The sputa were mixed with beef heart infusion broth, pH 7.4, and were either inoculated immediately into embryonated hen's eggs or were frozen in sealed glass ampoules and stored at -20° C until they could be examined for the presence of influenza virus. The maximum period of storage was approximately four months.

Acute and convalescent sera: Acute and convalescent blood specimens were obtained from 53 of the 69 patients. The acute specimens were collected soon after admission to the hospital, and the convalescent specimens were secured just prior to discharge, approximately six to ten days later. A third specimen was collected about five months later from 25 of the cases. Serum from these specimens was inactivated by heating for 30 minutes at 56° C, frozen in rubber stoppered glass ampoules and stored at -20° C until it could be examined by the red cell agglutination-inhibition technique for antibody activity against various known strains of influenza virus.

Autopsy material: Specimens of lung and bronchus were obtained at autopsy³ from 86 persons dying in Baltimore³ between December 1946 and October 1947. In addition one specimen was obtained from a person dying of pneumonia in May 1946. Of the 87 specimens, nine were from persons in whom death was believed to be due directly and primarily to pneumonia, 28 were from persons with pneumonia which was believed to be secondary to some other condition, and 50 were from persons who had no evidence of pneumonia. Classification was made on the basis of clinical and pathological data in the hospital and autopsy records. The group whose death was believed to be due primarily to pneumonia includes six cases of pneumococcal pneumonia and three cases which may not have been bacterial in origin since the causative organism was not identified. The group of secondary pneumonia consists of terminal or aspiration pneumonias and pneumonias first detected at autopsy. The third group includes only cases in which there was neither clinical nor pathological evidence of pulmonary pneumonia.

A 25 per cent suspension of the lung and bronchial tissue was made by emulsification with beef heart infusion broth in a Waring blender. The suspension was centrifuged to remove large particles of tissue and the supernatant was stored at -20°C in sealed glass ampoules until it could be examined for the presence of influenza virus. The maximum period of storage was approximately six months.

Virus isolation

Both sputum and lung specimens were examined for influenza virus by the same technique. Five hundred units of penicillin and 5000 μg . of streptomycin were added to each milligram of the specimen and about 0.4 ml. of this mixture was inoculated into the amniotic sac (29) of 12 or 13 day old chick embryos. Ten embryos per specimen were used for the first egg passage and six for each additional passage.

The embryos were incubated at 35°C for four days. On the fourth day amniotic fluid and embryos were harvested. The amniotic fluid was tested for the presence of influenza virus by the pattern agglutination technique (30) using chick and guinea pig red blood cells. If the fluid was negative by this test the lungs and tracheas of the embryos were removed and ground in a mortar with the homologous amniotic fluid. Penicillin and streptomycin were added to the mixture and this material was passed amniotically to 12 or 13 day old embryos. Three egg passages were carried out before a specimen was considered negative.

In an attempt to detect possible laboratory cross infection six embryos were inoculated with sterile broth at the same time each group of specimens was examined. The broth control was carried along with the specimens through three egg passages of six embryos each.

³ All autopsy specimens were obtained through the Department of Pathology of The Johns Hopkins Hospital and Medical School.

Identification of viruses

Amniotic fluids which gave positive tests by the pattern technique referred to above were inoculated into the allantoic sac of 10 or 11 day old chick embryos. Inoculated embryos were incubated for two days at 35°C , chilled overnight at 4°C , and the allantoic fluid harvested. Serial passage by the allantoic route was continued until allantoic fluid with a sufficient concentration of virus could be obtained for identification by the hemagglutination-inhibition test (31). A suitable antigen was usually obtained by the fourth egg passage.

Known influenza immune sera used in identification of viruses were prepared by subcutaneous inoculation of chickens with the following strains of influenza virus: the PR8 strain of influenza A, the Lee strain of influenza B, a strain of influenza A (F47A7)⁴ isolated from an uncomplicated case of influenza during the 1947 epidemic in Baltimore, a strain of influenza A (P47A7) isolated from the lungs of a case of pneumonia in January 1947, and a strain of swine influenza virus.

Some of the strains, particularly those isolated during March and April 1947, proved difficult to identify as either A or B using this technique. Three of these strains (P47A2, P47A7, P47A8) were selected for further study employing acute and convalescent sera from cases of influenza occurring during three different epidemics: the 1943 influenza A epidemic,⁵ the 1945 influenza B epidemic, and the 1947 influenza epidemic during which two of the three strains were isolated.

Serological tests

Serum was tested by the hemagglutination-inhibition technique (31) using known strains of influenza virus as antigen. The strains of influenza virus routinely used as antigen in these tests were the PR8 strain of influenza A, the Lee strain of influenza B, two recently isolated strains of influenza A (one [P47A2] from a fatal case of pneumonia occurring in January 1947 and one [F47A7] from an uncomplicated case of influenza in March 1947), a strain of influenza B (P47B2) from a case of pneumonia in January 1947, and a strain of swine influenza.⁶ In order to rule out the effects of variations in antigen concentration and variation in red cells used from time to time, all sera whose titers were to be compared were tested simultaneously.

⁴ The method of strain designation for influenza virus isolated in this laboratory is as follows: The first letter stands for the disease from which the virus was isolated (F = uncomplicated influenza; P = pneumonia). The first two numbers represent the year of isolation. The second letter refers to the type of influenza virus (A = influenza A; B = influenza B). The last number is the order of isolation in the laboratory. Thus F47A7 is the seventh strain of influenza isolated in this laboratory in 1947 from uncomplicated influenza.

⁵ These sera were obtained through the courtesy of Dr. Thomas Francis, Jr., Ann Arbor, Michigan.

⁶ Dr. Richard Shope, Princeton, New Jersey, kindly furnished his Strain 15.

TABLE I

Influenza virus isolation and serological data on specimens collected from clinical cases of bacterial pneumonia

Time of occurrence with respect to influenza	Virus isolation			Serological studies			Total		
	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.
Interepidemic	33	1	3	22	0	0	33	1	3
Epidemic	36	13	36	33	12	37	36	17	47

On the basis of serological results using the six antigens above, the cases of pneumonia were divided into four categories:

(1) Those in which the convalescent specimen when compared with the acute specimen showed a three-fold or more increase in titer to at least one of the strains of influenza A, but not to either strain of influenza B used as antigen.

(2) Those in which the convalescent specimen showed less than a three-fold increase in titer but in which the third specimen when compared with the convalescent specimen showed a four-fold or greater decrease in titer to at least one of the strains of influenza A, but not to either strain of influenza B.

(3) Those in which the third specimen when compared with the convalescent specimen showed at least a four-fold decrease in titer to both influenza A and influenza B antigens.

(4) Those in which there was neither a three-fold increase nor a four-fold decrease in titer to any of the antigens used in the test.

Since no specific increases or decreases in titer were obtained with the influenza B antigens it is not necessary to include these categories.

The cases in Group 2 were classified as pneumonia with positive serological evidence of recent or concomitant infection with influenza A. Specific decrease in titer over a period of months has previously been used (15) as the basis for serodiagnosis of infection with influenza virus, but the procedure is not so well established as that of diagnosis on the basis of specific increases in titer over a short period of time from the acute stage of the disease to early convalescence. In this laboratory it was observed that in a number of instances the convalescent specimen when compared with the acute specimen showed a non-specific decrease in titer to all the antigens employed in the test. Sometimes as much as a three- or four-fold decrease was noted. The cause of this non-specific decrease is entirely unknown. Since such a decrease would tend to mask specific increases in titer it would not give false positive results in category number 1. It would, however, tend to magnify specific decreases in titer if the third specimen were compared with the acute specimen. For this reason in making positive diagnoses on the basis of decreases in titer, the third specimen was always compared with the second, or with the convalescent specimen rather than with the acute specimen. Because of the uncertainties involved in diagnosis on the basis of specific decreases in titer over

a period of several months it was felt that only four-fold or greater decreases should be considered as serological evidence of infection with influenza virus.

The cases in Group 3 were classified as pneumonia with doubtful serological evidence of infection with influenza virus. Only two cases fell in this group. The significance of these doubtful results is difficult to determine. They may represent inherent inaccuracies in the test or they may be the result of anamnestic reactions. On the other hand, they may be indicative of actual infection with influenza virus. In the latter case their nonspecific nature might be explained either on the basis of infection with a strain of influenza virus with broad antigenic characteristics or on the basis of infection with more than one strain of influenza virus during the interval of several months.

The cases in Group 4 were classified as pneumonia with no serological evidence of infection with influenza virus.

RESULTS

Clinical cases of bacterial pneumonia

Isolation of virus

The results of both virus isolation and serological studies in the 69 cases of bacterial pneumonia investigated are shown in Table I.

Of the 33 specimens collected during the interepidemic period only one was positive by virus isolation. This was a specimen of sputum obtained on the fifth day of disease from a case of Type I pneumococcal lobar pneumonia occurring in January 1947. A strain of influenza B (P47B2) was isolated from this sputum on the first egg passage. Influenza virus was not isolated from a second sputum obtained on the seventh day of disease. On retesting the original sputum which had been stored at -20°C for two weeks influenza B was again isolated on the first egg passage. The other strain of influenza B (P47B1) was isolated from the lungs of a fatal case of pneumonia which also occurred in January of 1947. This case will be discussed later. During the interval between December 1945, at which time there was an epidemic of influenza B in Baltimore, and the time of isola-

TABLE II

Day of disease on which sputum was collected and virus isolated from clinical cases of bacterial pneumonia

Day of disease	Number of specimens			
	Inter-epidemic periods	Positive for influenza	Epidemic period	Positive for influenza
1-4	21		20	7
5-9	9	1	12	5
10 and over	3		3	1
Unknown			1	
Total	33	1	36	13

tion of these two strains of influenza B, approximately 100 specimens of nasal washings from persons with upper respiratory infections had been inoculated into chick embryos. In no instance was influenza virus detected.

Influenza virus was isolated from 13 of the 36 cases of bacterial pneumonia which occurred during the influenza epidemic in March and April, 1947. The cases from which these strains of virus were isolated were all classified as pneumococcal pneumonia. Two were bronchopneumonia and the others were lobar pneumonia. Six were shown to be due to Type I, two to Type XXV, one to Type II, one to Type IV, and two to untyped pneumococci. Both Type IV and Type XXII were present in the sputum in one case. In these 13 positive cases sputum specimens were obtained from the first to the 20th day after the onset of clinical symptoms of pneumonia (see Table II).

In this small series of cases there is no correlation between day of disease and ability to isolate virus from sputum. The fact that virus was isolated from six specimens on or after the fifth day of pneumonia is somewhat surprising because it is generally believed that the virus is demonstrable in nasal or throat washings from uncomplicated influenza only early in the disease.

The isolation of influenza virus beyond the expected time in these complicated cases of mixed virus and bacterial infection may be explained on the basis that the virus in conjunction with the pathogenic bacterium descends to the lower respiratory tract, persists and probably multiplies in the respiratory epithelium of the bronchial tree longer than it ordinarily persists in the epithelial cells of the upper respiratory tract.

Appropriate precautions were taken to prevent laboratory cross infections leading to false posi-

tive results. These are believed to have been adequate for the following reasons:

(1) All of the 13 strains isolated during the influenza epidemic were found to be similar to strains of influenza A isolated from uncomplicated cases of influenza occurring in Baltimore during the same period.

(2) In each case the influenza virus was isolated on the first egg passage.

(3) All of the strains upon original isolation showed certain characteristics of the "O" type virus (32) in that they had a much higher agglutinating titer for guinea pig red blood cells than for chicken red blood cells.

(4) Twelve of the positive sputa were retested after they had been stored at -20°C for approximately eight months. Virus was again isolated on the first egg passage from 11 of these. The 12th sputum was negative after two egg passages. It is believed that the long period of storage, during which time the specimens were allowed to thaw and refreeze at least once, might well account for the failure to reisolate influenza virus from this one case. The possibility that the first isolation of influenza virus from this sputum might have resulted from a laboratory cross infection must be considered. However, on original isolation this strain showed "O" characteristics, which usually do not persist beyond the first passage. Unfortunately, no second serum was obtained from this individual so that a confirmatory serological diagnosis could not be made.

(5) None of the 18 broth controls which were inoculated and passed in a manner similar to the sputum specimens was ever shown to be contaminated with virus.

(6) All strains gave low hemagglutination titers in the early egg passages which increased for five to six passages.

Serological studies

Acute and convalescent serum specimens were obtained from 22 of the 33 cases which occurred in the interepidemic periods. A third specimen was obtained from 10 of these. There were no positive serological results in this group. The three specimens of serum obtained from the one case which was positive by virus isolation gave negative results by all serological tests including the

test in which the homologous virus was used as antigen. This may be an indication that the influenza virus played no part in this particular patient's illness. However, it has been noted that a certain proportion of persons who have clinical influenza during proved influenza epidemics fail to show an antibody response (33-38). In this connection it is interesting to note that of 45 cases of uncomplicated influenza occurring during the 1947 epidemic from which virus was actually isolated, only 21 (39) showed a significant increase in titer when tested by the agglutination-inhibition technique. Therefore, it seems reasonable to believe that the influenza B virus might have played a part in the disease process even though it caused no increase in serum antibody content as measured by the hemagglutination-inhibition test.

There were two doubtful results obtained on the specimens in the interepidemic group. A case of clinical lobar pneumonia of undetermined cause, which occurred in February 1947, was tested serologically five months later and showed a four-fold decrease in titer to a current strain of influenza A as well as a six-fold decrease in titer to the Lee strain of influenza B. Another case of lobar pneumonia believed to be due to Type VIII pneumococcus, which occurred in July 1947, showed a decrease in titer to both influenza A and influenza B antigens after a period of three months.

Acute and convalescent serum specimens were obtained from 33 of the 36 cases which occurred during the 1947 epidemic of influenza A. A third specimen was obtained from 15 of these. Twelve positive serological results were obtained in these cases, 10 falling into Group 1 as described above, and two into Group 2. Of the 10 cases in which the convalescent specimen showed at least a three-fold increase to influenza A antigens seven were also positive by virus isolation. As previously stated these seven cases were pneumococcal pneumonia due to various types of pneumococci. The other three were also due to pneumococci. All three were classified clinically as lobar pneumonia. One was due to Type I and one to Type II. Both Type III and Type XI were present in the sputum of the third case. Of the two cases diagnosed serologically on the basis of a specific four-fold decrease, one was also positive by virus isolation. Both these cases were pneumococcal lobar pneumonia, Type I.

No doubtful results were obtained in the epidemic group. Twenty-three specimens, including three which were positive by virus isolation, were negative serologically.

Of the four strains of influenza A routinely used as antigen in the agglutination-inhibition test by far the greatest number of positive results were obtained with P47A2, a strain of influenza A isolated from the lungs of a fatal case of pneumonia which occurred in February 1947. Nine of the ten cases which fell into the first group of serological positives showed a three-fold or more increase in titer with this antigen. Seven of the ten were positive with F47A7, a strain of influenza A isolated from a clinical case of influenza in March 1947 and chosen in this laboratory as the reference 1947 influenza A strain. Only two of the ten showed a three-fold or more increase to PR8, and one showed a significant increase with a strain of swine influenza virus. Table III shows the results of ten serologically positive cases with the four influenza A antigens.

In summary, of the 33 cases in the interepidemic periods one was positive by virus isolation and none was positive by serological methods. Of the 36 cases in the epidemic period 13, or 36 per cent, were positive by virus isolation. Eight of these 13 were also positive by serological tests. In addition to these eight, four cases were positive by serological results but negative by virus isolation. Thus, a total of 17 cases, or 47 per cent of those occurring during the influenza epidemic, gave posi-

TABLE III

Ratio of convalescent serum titer to acute serum titer with four different influenza A antigens in cases of bacterial pneumonia with serological evidence of concurrent influenza virus infection

Specimen number	Convalescent titer/acute titer with various antigens			
	PR8	Swine	F47A7	P47A2
367	6.1	4.3	3.0	3.0
388	13.0	2.1	21.0	40.0
390	2.0	0.6	4.6	10.0
396	2.3	1.5	7.0	10.0
406	2.6	—	1.4	12.0
413	0.6	0.5	5.2	3.0
424	1.1	0.7	0.9	7.3
427	0.8	0.4	2.0	8.0
434	2.5	2.3	3.2	3.3
435	0.7	0.4	3.0	0.6
Total positive	2	1	7	9

tive evidence of the presence of influenza virus associated with lobar pneumonia. All of these 17 cases were pneumococcal pneumonia. Eight were classified as Type I, two as Type XXV and one each as Types II, IV and XXII. Both Types III and XI were present in the sputum of one and Types IV and XXII in the sputum of another. Two cases were due to pneumococci whose type was not determined.

Virus isolation from autopsy material

The results of virus isolation from autopsy material are shown in Table IV.

TABLE IV

Influenza virus isolations from specimens of lung and bronchus obtained at autopsy from patients with pneumonia and with no pneumonia

Classification	Epidemic period		Inter-epidemic period		Total	
	No. cases	No. pos.	No. cases	No. pos.	No. cases	No. pos.
Primary pneumonia	2	0	7	2	9	2
Secondary pneumonia	7	0	21	2	28	2
No pneumonia	26	0	24	0	50	0

Influenza virus was isolated from two of the nine fatal cases classified as primary pneumonia. Both pneumococcus Type II and a strain of influenza A were isolated from the lungs of one of these cases (P46A1) which occurred in May 1946. In order to determine the quantity of virus present in the lungs of this case, titrations were made by inoculating chick embryos both by the allantoic route and by the amniotic route. The dose which would infect 50 per cent of the embryos inoculated was calculated using the method proposed by Reed and Muench (40). The EID_{50} for 11 day chick embryos inoculated via the allantoic route was $10^{-7.8}$. For 13 day embryos inoculated by the amniotic method an EID_{50} of $10^{-8.3}$ was obtained. These data indicate that virus was present in large quantities.

At the time of occurrence of this case, clinical and epidemiological evidence indicated that influenza A had not been prevalent in Baltimore for 2½ years and laboratory studies on upper respiratory disease for at least six months prior to the case had not disclosed influenza A in the population.

In January, 1947, in Baltimore, one year after the last known clinical case of influenza B, a strain of influenza B was isolated from the lungs of another fatal case of pneumonia (P47B1) and pneumococcus Type XI was isolated from the tracheal exudate three days before death.

The clinical and pathological aspects of these two cases will be discussed in another paper (41).

Influenza virus was isolated from the lungs of two of the 28 cases classified as secondary pneumonia. Both of the cases occurred early in February, just one month prior to the influenza A epidemic in Baltimore. One of the two strains (P47A1) of virus isolated was similar to strains isolated from clinical cases of influenza A during the epidemic. The other strain (P47A2) was also influenza A but it differed antigenically from the epidemic strain.

Lack of data makes it impossible to determine the exact nature of the pulmonary involvement of these two cases. There is, however, evidence in both cases which indicates that there was some pulmonary involvement. Since these cases will not be included in the subsequent paper a brief resume of the clinical and pathological data is given here.

One of the patients (P47A1), a colored male 14 months old, entered Sydenham Hospital with a diagnosis of chicken pox from the Harriet Lane Home of The Johns Hopkins Hospital. On admission his temperature was 103, pulse 176 and respirations 44. The uvula, the tonsils and the posterior pharynx were injected. The lungs were clear. The patient was given penicillin. After four days the temperature was normal and the throat injection had subsided. A systolic murmur was present over the base of the heart. Subsequently, this became more pronounced, spreading to the apex. On the ninth hospital day the patient died. The final clinical diagnosis was heart failure of unknown etiology, healed varicella, otitis media, and acute pharyngitis.

At autopsy wide-spread collapse in the posterior parts of both lungs was noted. There were small scattered areas of what appeared to be consolidation. The right auricle and ventricle were much enlarged.

Sections of the lung showed a condition compatible with chronic passive congestion. There were also small scattered foci of coagulative necrosis. The fresher ones seemed to be composed of

fibrin, a few mononuclear cells and some hemorrhage. The walls of the alveoli in these areas were necrotic. These lesions were said to be not unlike those previously observed (42) in a case of congenital varicella.

Unfortunately, no cultures were made from this lung and it is impossible to determine what part, if any, pathogenic organisms might have played in the disease process. It is also impossible to determine whether or not influenza virus itself played any part in the formation of the lung lesions found at autopsy.

The other fatal case (P47A2) classified as secondary pneumonia from which a strain of influenza A was isolated was a white male seven months old. This patient was referred to Sydenham Hospital with a diagnosis of bronchopneumonia of one day's duration. He was dead on arrival at the hospital. A cisternal tap showed cloudy fluid containing *H. influenzae*. The patient's older brother who was brought to the hospital at the same time was subsequently shown to have had meningitis due to *H. influenzae*.

At autopsy the lungs were found to be mostly air-containing. The pleural surfaces were smooth. The bronchial mucosa was covered by thin mucoid

material. On section the cut surfaces of the lungs showed numerous grayish areas separated by areas of red. In some areas the foci of consolidation appeared to be around the walls of the small bronchi.

Histological examination of these lungs failed to show any evidence of consolidation, bronchitis, or interstitial pneumonia.

As in the previous case no cultures from the lungs were reported. A culture made in this laboratory revealed no significant pathogen but the plates were contaminated with a gram negative rod believed to be *Aerobacter aerogenes*.

Titration in chick embryos were made to determine the quantity of virus in this lung. The EID_{50} was $10^{-2.8}$ with 11 day embryos inoculated allantoically. This is a minimum figure since the titration was made after the specimen of lung had been stored for six months at $-20^{\circ}C$. During the storage period the specimen was known to have been thawed and refrozen at least once. The quantity of virus in the lung was therefore sufficient to suggest that the virus might have become established in the lung tissue. The role it played, either alone or with some undetermined bacterial agent,

TABLE V

Serological study of strains of influenza virus isolated from cases of bacterial pneumonia using serum from chickens immunized with known strains of influenza virus

Strain of virus	Source	Date of isolation	Hemagglutination-inhibition titer with specific immune chicken sera					
			PR8	Lee	Swine	F47A7	P47A2	P47A7
PR8			1670	128	182	120	338	388
Lee			69	588	169	42	45	417
Swine			52	32	1560	37	49	37
F47A7	Clinical influenza	March 1947	147	56	91	835	512	549
P46A1*	Fatal pneumonia	May 1946	182	32	112	64	779	256
P47B1	Fatal pneumonia	Jan. 1947	49	338	45	32	128	128
P47A1*	Fatal pneumonia	Feb. 1947	128	169	—	1175	—	—
P47A2	Fatal pneumonia	Feb. 1947	147	32	104	74	362	32
P47B2	Nonfatal pneumonia	Jan. 1947	32	169	32	32	32	32
P47A3*	Nonfatal pneumonia	March 1947	169	194	120	1350	—	—
P47A4	Nonfatal pneumonia	March 1947	208	256	208	779	56	512
P47A5	Nonfatal pneumonia	March 1947	128	64	79	588	169	223
P47A6	Nonfatal pneumonia	March 1947	294	182	182	1450	388	479
P47A7	Nonfatal pneumonia	March 1947	84	39	52	417	208	447
P47A8	Nonfatal pneumonia	March 1947	239	208	256	1560	512	512
P47A9	Nonfatal pneumonia	March 1947	182	256	60	779	417	223
P47A10	Nonfatal pneumonia	April 1947	208	112	158	722	239	417
P47A11	Nonfatal pneumonia	April 1947	223	194	120	958	479	447
P47A12	Nonfatal pneumonia	April 1947	779	1270	512	2200	891	588
P47A13	Nonfatal pneumonia	April 1947	138	338	169	512	588	294
P47A14	Nonfatal pneumonia	April 1947	91	104	52	512	294	128
P47A15	Nonfatal pneumonia	April 1947	97	79	32	388	182	91

* All except these tests were run simultaneously.

in the production of the clinical symptoms cannot be determined on the basis of available data.

Strain identification

The results of hemagglutination-inhibition tests using known immune chicken sera with the various strains of virus isolated from sputum or lung specimens are shown in Table V.

Interpretation of these data is very difficult since the homologous titers of the various immune sera were different and since there was some unavoidable variation in the quantities of the different antigens used in the test.

It seems clear, however, that of the four strains isolated from the lungs of fatal cases of pneumonia one (P47B1) was a typical strain of influenza B and two (P46A1 and P47A2) were strains of influenza A which immunologically were very similar to each other, and somewhat similar to the PR8 strain of influenza A. Neither of these strains showed cross reactions with the Lee strain of influenza B. The fourth strain (P47A1) was inhibited to almost the same extent by both PR8 chicken antiserum and Lee chicken antiserum. It was inhibited by much higher dilutions of chicken antiserum to the strain chosen in this laboratory because of its early isolation and its antigenic similarity to subsequently isolated strains as the reference 1947 strain of influenza A (F47A7).

Of the 14 strains isolated from the sputum of nonfatal cases of pneumonia, one (P47B2) was a typical strain of influenza B. The other 13 strains, all isolated in March and April, 1947, were similar to each other and to the reference 1947 strain of in-

fluenza A (F47A7). They were inhibited to almost the same extent both by PR8 chicken antiserum and by Lee chicken antiserum.

The tests described above were carried out after the various strains had been passed only three or four times in chick embryos. Since it is known that antigen prepared from recently isolated strains may contain a substance which inhibits the agglutination of red blood cells in the presence of normal as well as immune sera (57) three strains (P47A2, P47A8 and P47A7) were selected for further study after repeated passages in chick embryos. After 12 embryo passages, the results were essentially the same as those obtained using antigens prepared from earlier egg passage material. Strain P47A2 was again inhibited, although to a small extent, by PR8 antiserum but not by Lee antiserum. Strains P47A7 and P47A8 were inhibited to almost the same extent by both PR8 antiserum and by Lee antiserum. They were inhibited to a greater extent by F47A7 antiserum.

By using the unidentified strains of virus as antigens in hemagglutination-inhibition tests with human acute and convalescent sera obtained from persons who had clinical influenza during three different proved influenza epidemics, somewhat more clear-cut results were obtained. The results of these tests are shown in Table VI.

On the basis of the results obtained with human acute and convalescent sera, the strains of influenza virus isolated from patients with pneumonia during the 1947 epidemic of influenza in Baltimore are believed to be more closely related to influenza A than to influenza B and to be very closely related to strains of influenza virus isolated

TABLE VI
Identification of selected strains of influenza virus using human acute and convalescent sera from three influenza epidemics

Serum specimen number	Source of serum	Convalescent titer/acute titer with various antigens					
		PR8	Lee	P47A2	F47A7	P47A7	P47A8
1	1943 influenza A epidemic	5.0	1.2	5.2	1.2	1.6	1.1
2	1943 influenza A epidemic	8.5	1.8	13.1	2.5	3.2	1.2
3	1943 influenza A epidemic	2.1	1.1	5.0	2.6	2.6	2.8
4	1943 influenza A epidemic	9.1	1.1	17.3	2.3	4.6	2.3
5	1945 influenza B epidemic	1.3	5.7	1.3	0.7	0.7	1.0
6	1945 influenza B epidemic	1.3	16.0	1.2	1.0	1.2	1.0
7	1945 influenza B epidemic	1.3	24.0	1.6	1.1	1.1	0.8
8	1947 influenza A epidemic	10.0	1.0	16.0	5.3	9.2	8.0
9	1947 influenza A epidemic	3.5	1.2	60.0	10.0	7.0	9.3
10	1947 influenza A epidemic	7.0	1.4	3.7	5.7	5.3	8.0

from uncomplicated cases of influenza occurring during the same period.

DISCUSSION

It seems evident on the basis of results obtained in these and other similar studies that often during influenza epidemics and occasionally during non-epidemic periods, bacterial pneumonia is in some way related to recent or concurrent infection with influenza virus. The pathogenesis of these multiple infections is not yet understood.

It seems unlikely that influenza virus alone is often responsible for severe pneumonia. During the 1918 pandemic a vast number of fatal cases of pneumonia were examined (4, 43-47) and in only two reported instances (47) was it impossible to isolate a pathogenic organism from the lungs. Since the discovery of the virus etiology of influenza a number of successful attempts have been made to isolate the virus from the lungs of fatal cases of pneumonia. In all but one of these (18) a pathogenic organism was also present in the lungs.

No pathogenic organism was isolated from the lungs of one of the fatal cases reported in detail in another paper (41) and the pathology in this case is that of pure virus pneumonia. A pneumococcus Type XI was isolated, however, from the tracheal exudate three days before the patient died of bronchopneumonia.

Influenza virus has been isolated from the sputum of non-fatal cases of pneumonia. In most instances the predominating organisms in the sputum from these patients were pneumococci or hemolytic staphylococci. However, there is clinical evidence that infections with influenza virus alone can cause slight abnormal pulmonary signs and might actually cause some patchy consolidation (22, 48). Reimann (49) in a review of pneumonias of viral origin states that pneumonia is an integral part of influenza but for unknown reasons influenzal lesions of the lung are more often invaded by pathogenic organisms than are the lesions of other viral pneumonias.

Probably the most generally accepted explanation concerning the relationship of influenza virus and bacteria in the pneumonias accompanying influenza is that the viral agent plays only a preparatory role. It is commonly believed that debilitating infections such as influenza decrease the general resistance of the host and predispose to bacterial

invasion. The mechanism by which general host resistance is lowered is not understood. A more clear-cut explanation for the lowering of local resistance in the respiratory tract can be given. It is known that influenzal infections in the ferret (50) and probably in the human (51) cause destruction of the ciliated epithelium of the respiratory tract. The destruction of this important local defense mechanism would be expected to facilitate invasion of the lungs by pathogenic organisms. Symptoms of pneumonia often have their onset a week or more after the first symptoms of influenza.

Although there is only slight evidence (26) to support such a concept, there is a possibility that the virus might act on certain potentially pathogenic organisms composing the normal flora of the nasopharynx in such a way as to increase their virulence and invasive ability. The invasive ability might be enhanced simply by the protective action against phagocytosis offered by mucus present as a result of influenzal infection. As reported herein, the isolation of influenza virus from 36 per cent of bacterial pneumonias occurring during an influenza epidemic indicates that the virus could have been playing an active part in the disease despite the onset of the pneumonia several days after the onset of clinical influenza. Both agents may be actively engaged in some as yet unknown relationship. Pathological manifestations of virus and bacterial complex infections are often similar regardless of the type of bacterium. The virus is thought to play a part in producing the pathological lesions and superimposed on such lesions are changes brought about by the bacterium.

Taylor (52) observed that a sublethal dose of influenza virus kills mice if followed in a day or two by intranasal instillation of sterile fluid. He proposed that the sterile fluid upset a delicate host-parasite relationship between spread of the virus and antibody production. By analogy such a mechanism might explain the simultaneous bacterial and influenza infections in man.

The inflammation produced by the bacterium may enhance release of the influenza virus from infected cells and aid in the spread of the virus to uninfected cells before the production of sufficient antibodies could prevent such a spread.

It is interesting to note that in five instances in the present study influenza virus was isolated from cases of pneumonia during interepidemic pe-

riods with respect to influenza. Sporadic cases of influenza have been reported by several investigators (20, 53-56) but there is not yet general agreement concerning the whereabouts of influenza virus between epidemic periods. Further investigation of bacterial pneumonias during periods when uncomplicated cases of influenza are not occurring to any great extent might indicate that influenza is indeed an endemic disease reaching epidemic proportions at more or less regular intervals. Why it was possible to isolate influenza virus from the sputum or lung of five cases of pneumonia and not possible to demonstrate the virus in nasal washings in any of about 100 cases of uncomplicated upper respiratory infections during the same interepidemic periods is not known. It may be that in cases of complex infection the virus descends to the lower respiratory tract and proliferates longer than it would in the upper respiratory epithelium. The number of uncomplicated cases from which nasal washings were examined represents only a small fraction of all the upper respiratory infections which occurred during the interepidemic periods and it is possible that more extensive studies would reveal sporadic cases of uncomplicated influenza as well as influenza complicated by pneumonia.

While these studies have been concerned with the simultaneous occurrence of influenza virus and bacteria in pneumonia, it is difficult to resist the speculation that other respiratory viruses, if and when techniques are available for their ready isolation and identification, will also be found in association with bacteria in other cases of pneumonia in man.

SUMMARY

1. Seventeen of 36 cases of pneumococcal pneumonia occurring during an epidemic of influenza A in Baltimore in March and April, 1947, yielded positive evidence, either by virus isolation or by serological studies of recent or concurrent infection with influenza virus.

2. All of the 13 strains of influenza virus isolated from these 36 cases were found to be similar to strains of virus isolated from uncomplicated cases of influenza occurring during the same period.

3. Influenza virus was isolated from the sputum of one of 33 cases of bacterial pneumonia oc-

curing during interepidemic periods. The one positive specimen was from a case of pneumococcal lobar pneumonia and the virus isolated was a typical strain of influenza B.

4. The lungs of two fatal cases of primary pneumonia and two cases of secondary pneumonia, in which no pathogenic organism was identified, yielded influenza virus. Three of the strains of virus were influenza A and one was influenza B. All four of these cases occurred during interepidemic periods with respect to influenza.

5. These studies indicate that influenza virus plays a role in the etiology of certain pneumonias which have hitherto been considered primarily bacterial in origin.

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DETERMINATION OF THE PLASMA GLYCINE AFTER GELATIN FEEDING AS A DIAGNOSTIC PROCEDURE FOR PANCREATIC FIBROSIS

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A retardation of the absorption of amino acids derived from ingested protein into the bloodstream is known to result from a failure of the secretion of the pancreatic proteinases. West, Wilson and Eyles (1) have shown that a much smaller rise in blood amino nitrogen occurs in children with pancreatic fibrosis than in normal children after a test meal of casein. Preliminary results obtained by these authors indicated that gelatin might be a better test protein. To avoid a choice between the technically somewhat complex manometric ninhydrin procedure (Hamilton and Van Slyke [2]) and the analytically unsatisfactory colorimetric amino nitrogen methods (cf. Chinard and Van Slyke [3]) we have determined the plasma concentration of the single amino acid, glycine. Advantage is taken of the convenient method available for glycine (Alexander, Landwehr and Seligman [4]), the high glycine content of gelatin (25.5%), and the relatively slow disposition of this amino acid. Comparison of the fasting plasma glycine with the level 150 minutes after gelatin feeding provides a simple diagnostic procedure, although some qualifications will be noted below.

Although duodenal intubation in most cases is not a difficult procedure, upon occasion it may be troublesome. Mention might be made of a) several instances where four or five separate unsuccessful attempts were made to obtain a satisfactory specimen from a patient, b) the undesirability of the procedure in the severely ill infant, and c) the occasional febrile reaction following intubation. The present procedure offers an alternative diagnostic test.

EXPERIMENTAL

The test procedure is as follows: the patient is given, six to 12 hours after the last meal, 1.5 grams of protein per kilogram of body weight in the form of Knox gelatin, in 30 ml. of warm water per kilograms, either by mouth or by gavage. Venous blood is collected just

before the feeding and 2½ hours after. The plasma is separated *at once*, deproteinized with picric acid, and the glycine determined according to Alexander *et al.* (4). The analysis depends upon a colorimetric measure of the formaldehyde released from glycine by ninhydrin. A comparison of the optical densities of the two samples without translating them into glycine concentrations is sufficient.

For the deproteinization, 5 volumes of picric acid are added to the plasma, and the mixture centrifuged (Hamilton and Van Slyke [2]). When analyzing fasting blood, a 4 ml. aliquot of plasma filtrate is used; for the samples taken later, 2 ml. usually give a suitable depth of color. This means that 3 or 4 ml. of fasting blood and 1.5 ml. of the postprandial blood samples permit duplicate analyses. The requirements can be decreased if necessary to about 0.5 ml. of blood by cutting quantities used in half throughout the procedure, and distilling over to a volume of 5 ml. rather than 10 ml. A drop of normal sodium hydroxide is added for each 2 ml. of picric acid filtrate used in the determination, to neutralize the picric acid.

The patients studied ranged from one month to 12 years in age. In most cases the plasma glycine was determined before the test, and one and 2½ hours after the test meal. The total α -amino nitrogen in many cases was also determined for comparison.

RESULTS AND DISCUSSION

Three groups of patients were studied:

1. Twenty-four patients for whom the diagnosis of pancreatic fibrosis had been established, in 22 of them by the absence of tryptic activity in the duodenal contents.

2. Nineteen patients for whom the diagnosis of pancreatic fibrosis had been excluded. These were malnourished children, several of whom suggested the diagnosis of pancreatic fibrosis on clinical grounds.

3. Six patients whose clinical course was consistent with the diagnosis of pancreatic fibrosis, who had, however, definite although diminished tryptic activity.

TABLE I

The plasma glycine nitrogen following gelatin feeding
The values are averages for the group of patients indicated, and are in mg. N per 100 ml.

	Fast-ing	In-crease after 60 min.	In-crease after 150 min.	Ratio, 150 min. to fasting	
				Mean	Range
Controls (19)	0.26	0.90*	1.28	5.0	2.6 to 7.6
Pancreatics (24)	0.32	0.44†	0.59	1.9	1.2 to 3.2
Pancreatics + pan-creatin (8)	0.33	1.37	1.18	3.6	2.6 to 5.3

* Average includes only 13 cases.
† Average includes only 14 cases.

To the first two groups of patients the following generalization could be applied, with two excep-tions to be discussed later.

Patients without tryptic activity have their plasma glycine increased up to 2.5 times the fasting level (mean, 1.9 times) ; patients with good tryptic activity have their glycine increased by 2.6 to 7.6 times (mean, 5.0 times) 150 minutes after the test meal (Table I).

Similarly these patients could be divided into the same two groups by the rise in the plasma α -amino nitrogen in the 17 cases where this deter-mination was made (Table II). Seven patients given pancreatin (coated granules) with the gela-tin had their glycine and α -amino nitrogen toler-ance curves moved from the deficient to the high normal range. The results were similar whether gelatin was fed by mouth or introduced by tube directly into the stomach or duodenum.

Included in the group of 24 patients with no sig-nificant pancreatic enzymatic activity, were three patients showing rather high glycine responses, one so high on two of three occasions as to represent a striking exception. These patients all, inci-

dentally, had a diagnosis of meconium ileus at birth. The ratios of the plasma glycine levels after and before gelatin were: R. E., 2.5; M. M., 3.2; and D. B., 4.1, 5.7, and 3.0. The last patient had hepatic disease (probably homologous serum jaun-dice) more severe at the time of the first two tests than at the time of the third test. Since the plasma glycine level undoubtedly represents a balance be-tween the relative rates of the absorption and of the disposal of glycine, severe hepatic disease may be a complicating factor in this test.

The third group consisted of the six patients who presented relatively low yet quite definite tryptic activity in their duodenal contents. The ratio of final to original glycine levels were as fol-lows: D. R., 2.2; A. O., 2.4; S. F., 2.9; F. M., 3.2; R. V., 3.8; P. N., 5.7. Three of these values are above the range obtained for patients having no significant amount of tryptic activity. That a group of children showing intermediate degrees of pancreatic enzyme activity should range rather widely in their rates of gelatin digestion is not surprising. Heretofore partial pancreatic insuf-ficiency, in patients with the clinical appearance of pancreatic fibrosis, has not been described. This group is under study.

Finally, a single patient was observed who had normal enzymatic activities in his duodenal fluid, but who showed a rise to only 1.9 times his fast-ing plasma glycine after his test meal. This pa-tient could absorb glycine no better when pan-creatin was fed with the gelatin, or even when the glycine was fed *in the free state*. Since children with pancreatic disease absorbed glycine readily in either of the last two situations, clearly this is a different type of deficiency, and accordingly this patient is not included in the general averages.

TABLE II

The plasma α -amino nitrogen following gelatin feeding
The concentrations are in milligrams per 100 ml.

	Fasting		Increase				Ratio, higher level to fasting level	
			After 60 min.		After 150 min.			
	Mean	Range	Mean	Range	Mean	Range	Mean S.D.	Range
Controls (10)	3.55	2.90 to 4.54	2.31	0.60 to 4.48	3.51	1.49 to 6.22	2.0±0.3	1.43 to 2.50
Pancreatics (7)	3.38	2.89 to 4.05	0.32	-0.37 to 1.10	0.57	-0.78 to 1.35	1.2±0.2	0.93 to 1.40
Pancreatics + pan- creatin (6)	3.23	2.55 to 4.81	4.04	3.02 to 5.35	3.65	2.10 to 5.33	2.3±0.4	1.82 to 2.92

The subject also yielded a flat glucose tolerance curve.

Apparently a further test is desirable for an occasional patient to determine whether or not pancreatin intensifies significantly the elevation of the plasma glycine produced by gelatin feeding. Not only will this permit detection of the probably rare patient who cannot absorb readily even free glycine, but should also assist in clarifying the status of subjects who may show equivocal responses.

SUMMARY

1. Forty-nine patients presenting symptoms of malnutrition and respiratory disease suggesting a diagnosis of pancreatic fibrosis have been studied as to the response of their plasma glycine concentrations to the feeding of gelatin.

2. Twenty-two out of 24 patients having no significant tryptic activity in their duodenal fluid showed rises to not more than 2.5 times their fasting glycine levels. Nineteen patients having good pancreatic enzyme activity showed increases to more than 2.5 times the fasting value (average, five times fasting level). Liver disease may have been a factor in the exceptional increases shown by one of the patients with pancreatic disease.

3. The feeding of pancreatin with the gelatin changed the responses of either the glycine or α -

amino nitrogen from the pancreatic-deficient range to the high normal range.

4. A group of six patients showing definite, although small, tryptic activity in their duodenal fluids gave rather widely ranging responses to the test.

5. A single patient presenting evidence of normal secretion of pancreatic enzymes absorbed glycine poorly even when the amino acid was fed in the free state.

The assistance of Miss Jean Streicher is gratefully acknowledged.

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STUDIES IN CIRRHOSIS OF THE LIVER. I. RELATIONSHIP BETWEEN PLASMA VOLUME, PLASMA PROTEIN CONCENTRATIONS AND TOTAL CIRCULATING PROTEINS

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INTRODUCTION

Addis, Poo and Lew (1) aptly demonstrated the importance of the liver as a storage place for protein substances. The extensive studies of Whipple and his associates (2, 3) allowed some insight into the magnitude of this protein storage and its availability during time of need. It also seems well established that the liver is the principal site of formation of albumin and fibrinogen and to a lesser extent of globulin (4-10). In the presence of severe hepatic disease such as advanced cirrhosis, the functions of protein synthesis and storage are presumably inhibited and this inhibition is reflected by alterations in the various protein concentrations in the plasma. Supporting this concept are the observations (4, 5) which correlated the fibrinogen content of the blood with the degree of liver injury. Similarly, hypoalbuminemia has been established as a characteristic laboratory finding in patients with far advanced cirrhosis of the liver (6, 7). Plasma protein concentrations cannot, however, be used as an index for total circulating proteins or plasma volume (11, 12). It has already been shown that the plasma volume may be elevated in patients with cirrhosis of the liver (13). In a previous communication (14), data were presented from patients with cirrhosis of the liver, illustrating normal values for total circulating proteins despite the presence of low protein concentrations in the serum. The purpose of this communication is to record further data concerned with the plasma volume and the quantitative estimation of the various plasma proteins in patients with far advanced Laennec's cirrhosis of the liver.

MATERIAL AND METHODS

Twelve patients were chosen for this study. At the onset of investigation each patient exhibited edema, ascites, anemia, hepatomegaly, spider angiomas, and

evidence of protein and multiple vitamin deficiency. Patients with jaundice were excluded from the study. Biopsy specimens from the liver, obtained by means of peritoneoscopy from 10 of the patients, were consistent with the impression of far advanced Laennec's cirrhosis. The presence of complicating disease was excluded by means of venous pressure determinations, circulation times, electrocardiograms, renal function tests and roentgenograms of the entire gastrointestinal tract and chest. All patients were men and ranged in age from 38 to 67 years. The average age for the entire group was 49.7 years.

Laboratory tests including hippuric acid excretion (intravenous procedure), bromsulphthalein excretion (5 mg. per kilogram of body weight), prothrombin time, cephalin-cholesterol flocculation and quantitative thymol turbidity were repeated at approximately biweekly intervals. As far as possible these laboratory procedures were obtained within 48 hours before plasma volumes and protein concentrations were determined.

Plasma volumes were determined in the fasting state at bed rest according to the method of Gibson and Evans (15) as modified by Gibson and Evelyn (16). Dye free samples of serum obtained before the injection of T-1824 were used as control readings. Fifteen minutes was found sufficient time to permit thorough mixing of the dye. Plasma volume was finally estimated by extrapolation of the time-concentration curve of the dye. Predicted plasma volumes were estimated according to height (17). Total blood volume was calculated from the simultaneous determination of the plasma volume and the venous hematocrit according to the formula (18):

$$\frac{\text{Plasma volume}}{100 - \text{hematocrit}} \times 100 = \text{blood volume.}$$

The venous hematocrit was determined according to the method of Wintrobe modified by the use of a 4 ml. capacity tube.

Total protein and albumin were determined according to the method of Howe (19) as modified by the use of Conway cells for the estimation of ammonia nitrogen (20). Fibrinogen was determined by conversion to fibrin (21) and, after digestion, the estimation of fibrin nitrogen by means of the Conway cell procedure. All blood samples for nitrogen determinations, hematocrits and plasma volumes were obtained simultaneously.

During the period of observation each patient was maintained on a daily diet in excess of 3000 calories,

120 or more grams of protein, moderately low in fat and containing approximately 2.7 grams of sodium chloride. Dietary therapy was augmented by vitamin supplements and, in some patients at the beginning of the study, by choline chloride and yeast. In a very few instances protein hydrolysates were given for a short period of time. For approximately the first half of the period of observation each patient received ammonium chloride, 6 grams daily for three days, followed by the administration of 2 ml. of mercupurin or mercurhydrin either intra-

venously or intramuscularly. After a three day rest period this procedure was repeated. For reasons to be discussed it was felt that diuresis in this manner did not appreciably influence the plasma volume. All patients were considered semi-ambulatory.

RESULTS

Persistently abnormal results for thymol turbidity, cephalin-cholesterol flocculation, hippuric

TABLE I

Correlated data obtained from observations on plasma volume, blood volume, serum protein concentrations and total circulating proteins in 12 patients with advanced Laennec's cirrhosis of the liver

Day	Weight	Plasma volume	Hemato-crit (Average)	Total protein	Albumin	Globulin	Fibrinogen	Total circulating protein	Total circulating albumin	Total circulating globulin	Total circulating fibrinogen
	kg.	ml.		gm./100 ml.	gm./100 ml.	gm./100 ml.	mg./100 ml.	grams	grams	grams	grams
J. C.	66.3*	2750*		6.00†	4.00†	2.00†		165.0†	110.0†	55.0†	5.5†
1	—	2630	38.0	5.14	2.23	2.91	365	135.2	58.6	76.6	9.6
12	68.1	3740	40.0	5.70	2.83	2.86	246	213.2	105.9	107.3	9.2
32	66.3	5010	36.5	6.80	3.20	3.60	170	340.9	160.3	180.4	8.5
46	66.8	5130	38.5	6.70	3.10	3.60	168	343.7	159.0	184.7	8.6
56	68.1	5180	35.5	6.01	4.30	1.71	113	311.3	222.7	88.6	5.9
63	68.4	4910	32.2	6.95	3.22	3.73	505	341.2	158.1	183.1	24.7
88	70.4	4350	41.0	6.59	3.52	3.07	235	286.7	153.1	133.6	10.2
125	71.3	4250	38.0	6.83	3.78	3.05	296	290.3	160.7	129.6	12.5
179	65.9	5540	39.0	7.01	4.05	2.96	234	388.4	224.4	164.0	12.9
217	—	5150	39.0	7.00	3.25	3.75	189	360.5	167.4	193.1	9.7
245	62.2	5080	40.0	6.40	4.60	1.80	72	325.1	233.7	91.4	3.6
259	64.7	4980	39.0	6.37	3.59	2.78	164	317.2	178.8	138.4	8.1
T. M.	74.0*	2970*						178.2†	118.8†	59.4†	5.9†
1	70.9	3910	39.0	3.54	1.59	1.95	188	138.4	62.2	76.2	7.3
15	61.8	3460	40.5	5.00	—	—	208	173.0	—	—	7.2
28	59.5	4820	36.0	5.80	3.46	2.34	346	279.6	166.8	112.8	16.7
45	65.4	4050	33.5	5.44	2.14	3.30	344	220.3	86.7	133.6	13.9
59	57.2	3120	36.0	5.75	2.22	3.53	530	179.4	69.3	111.1	16.5
73	60.9	3830	34.5	5.55	2.26	3.29	415	212.6	86.6	126.0	15.8
87	61.3	3840	29.0	4.25	1.49	2.76	472	163.2	57.2	106.0	18.1
107	62.9	4010	33.0	5.60	—	—	457	224.6	—	—	18.3
147	65.6	3380	32.0	5.09	2.53	2.56	425	172.0	85.5	86.5	14.3
C. P.	73.6*	3080*						184.8†	123.2†	61.6†	6.1†
1	—	3480	—	6.28	2.49	3.79	492	218.5	86.6	131.9	17.1
25	72.5	3310	40	6.90	3.48	3.42	—	228.4	115.2	113.2	—
84	71.3	2900	48	7.59	4.82	2.77	283	220.1	139.8	80.3	8.2
F. P.	67.7*	2778*						166.7†	111.1†	55.6†	5.5†
1	65.9	4820	32	4.99	1.82	3.17	410	240.5	87.7	152.8	19.7
22	67.9	4410	33	—	3.68	—	532	—	162.3	—	23.4
61	64.5	4460	35	5.23	2.83	2.40	452	233.2	126.2	107.0	20.1
92	61.8	4040	32	6.27	2.74	3.53	315	253.3	110.7	142.6	12.7
110	60.4	3965	32	6.82	2.27	4.55	315	270.4	90.0	180.4	12.4
J. G.	67.7*	2805*						168.3†	112.2†	56.1†	5.6†
1	71.3	4890	33.5	5.26	2.30	2.96	202	257.2	112.5	144.7	9.9
15	68.1	4260	35.7	7.48	3.21	4.27	346	318.7	136.8	181.9	14.7
33	68.1	4150	38.7	7.60	3.60	4.00	225	315.4	149.4	166.0	9.3
43	71.3	4470	38.5	7.31	3.58	3.73	322	326.7	160.0	166.7	14.4
147	73.1	4835	41	7.04	4.65	2.39	357	340.4	224.8	115.6	17.2
209	74.3	—	37	6.13	3.26	2.87	—	—	—	—	—
235	71.3	4880	38	6.58	3.40	3.18	283	321.1	165.9	155.2	13.8

* Predicted normal weight and plasma volume for each patient.

† Accepted minimum normal values for total protein, albumin and globulin concentrations.

‡ Minimum normal quantity of total circulating protein, albumin, globulin and fibrinogen for each patient.

TABLE I—Continued

Day	Weight	Plasma volume	Hemato- crit (Average)	Total protein	Albumin	Globulin	Fi- brinogen	Total circu- lating protein	Total circu- lating albumin	Total circu- lating globulin	Total circu- lating fibrinogen
	kg.	ml.		gm./ 100 ml.	gm./ 100 ml.	gm./ 100 ml.	mg./ 100 ml.	grams	grams	grams	grams
G. O.	67.7*	2750*						165.0†	110.0†	55.0†	5.5†
1	73.6	3375	40	6.72	1.48	5.24	359	226.8	49.9	176.9	12.1
3	—	3450	39	6.42	2.72	3.70	195	221.5	93.8	127.7	6.7
11	—	3320	31	3.72	1.66	2.06	391	123.5	55.1	68.4	12.9
23	86.3	4620	34	5.75	2.83	2.92	283	265.7	130.8	134.9	13.0
36	87.5	5530	36.5	6.44	3.43	3.01	303	356.1	189.7	166.4	16.7
60	82.7	4960	34.5	4.78	2.44	2.34	314	237.1	121.0	116.1	15.5
74	85.9	3950	31.1	6.15	2.54	3.61	376	242.9	100.3	142.6	14.8
85	87.2	—	—	6.84	3.22	3.62	302	—	—	—	—
88	81.8	4790	33.7	6.65	1.97	4.68	282	318.5	94.3	224.2	13.5
109	84.3	5190	36	5.95	3.83	2.12	472	308.8	198.8	110.0	24.5
122	80.0	4250	37	5.76	3.35	2.41	357	244.8	142.4	102.4	15.1
136	90.0	4620	38	6.60	2.46	4.14	—	304.9	113.6	191.3	—
176	77.7	3940	39	5.89	1.14	4.75	170	232.1	44.9	187.2	6.6
192	84.3	4100	38	5.88	2.46	3.42	378	241.1	100.9	140.2	15.4
218	80.0	4000	38	5.44	2.70	2.74	375	217.6	108.0	109.6	15.0
232	—	4040	38	5.72	2.26	3.46	425	231.1	91.3	139.8	17.1
276	84.5	3905	35	7.72	2.08	5.64	315	301.4	81.2	220.2	12.3
280	86.5	3820	38	6.13	2.36	3.77	440	234.1	90.1	144.0	16.8
A. D.	83.1*	3223*						193.4†	128.9†	64.5†	6.4†
1	80.9	4040	44.5	4.45	2.45	2.00	315	179.8	99.0	80.8	12.7
8	86.3	3810	38	5.75	3.37	2.38	189	219.1	128.4	90.7	7.2
12	81.6	4060	44	5.18	3.02	2.16	189	210.3	122.6	87.7	7.6
22	82.2	3320	43	6.02	3.02	3.00	312	199.9	100.3	99.6	10.3
35	84.7	3915	43	5.64	2.36	3.28	315	220.8	92.4	128.4	12.3
43	84.3	4400	42	—	—	—	—	—	—	—	—
64	85.9	4550	41	5.00	2.35	2.65	126	227.5	106.9	120.6	5.7
78	—	4058	45	5.94	3.30	2.64	420	241.0	133.9	107.1	17.0
H. R.	74.0*	2981*						178.8†	119.2†	59.6†	5.9†
1	—	2622	37	6.55	1.40	5.15	127	171.7	36.7	135.0	3.3
14	81.8	2278	40	5.93	1.21	4.72	99.8	135.1	27.6	107.5	2.2
15	74.0	3320	40.5	6.57	2.33	4.24	255	218.2	77.3	140.9	8.4
28	78.6	3900	38	6.25	1.48	4.77	238	243.7	57.7	186.0	9.2
49	70.4	3820	39	7.10	1.32	5.78	169	271.2	50.4	220.8	6.4
63	80.2	3990	38	6.61	2.88	3.73	217	263.7	114.9	148.8	8.6
C. W.	74.0*	2987*						179.2†	119.5†	59.7†	5.9†
1	cast	2920	40	5.22	2.16	3.06	299	152.4	63.1	89.3	8.7
10	cast	3005	40	6.72	3.85	2.87	378	201.9	115.7	86.2	11.3
31	cast	3440	40	7.72	1.25	6.47	236	265.6	43.0	222.6	8.1
58	61.5	2940	38	7.05	4.54	2.51	441	207.2	133.4	73.8	12.9
62	61.5	3270	38	7.72	4.30	3.42	520	252.4	140.6	111.8	17.0
94	63.1	3030	39	6.83	3.96	2.87	—	206.8	119.9	86.9	—
J. R.	70.9*	2926*						175.5†	117.0†	58.5†	5.8†
1	84.5	5270	24	6.44	1.52	4.92	163	339.4	80.1	259.3	8.5
58	82.0	4980	36	5.64	—	—	220	280.9	—	—	10.9
106	81.8	4740	36	3.41	2.08	1.33	284	161.6	96.6	63.0	13.4
142	83.6	4500	40	5.80	2.08	3.72	189	261.0	93.6	167.4	8.5
170	82.5	—	39	6.40	2.83	3.57	252	—	—	—	—
184	83.6	—	36	8.62	2.83	5.79	126	—	—	—	—
205	82.2	4440	—	—	—	—	—	—	—	—	—
E. D.	73.1*	3003*						180.1†	120.1†	60.0†	6.0†
1	70.9	3130	40	6.12	3.77	2.35	409	191.6	118.0	73.6	12.8
9	70.9	4230	44	7.25	2.46	4.79	365	306.7	104.1	202.6	15.4
23	72.2	3950	45	7.94	3.78	4.16	208	313.6	149.3	164.3	8.2
36	72.7	3620	44	6.78	1.70	5.08	567	245.4	61.5	183.9	20.5
A. B.	66.3*	2772*						166.3†	110.9†	55.4†	5.5†
1	—	2710	41	6.65	1.82	4.83	270	180.2	49.3	130.9	7.3
15	—	2300	41.5	7.31	3.15	4.16	315	168.1	72.4	95.7	7.2

acid excretion and bromsulphthalein excretion were found in all patients throughout the study. Prothrombin time returned to normal in all patients soon after the institution of parenteral and oral vitamin K therapy. The results of these five tests were taken to indicate that persistent liver damage was present in each patient for the duration of the study.

Table I contains the correlated data obtained from observations on plasma volume, serum protein concentrations and total circulating proteins in the 12 patients. Percentage deviation values for the various serum protein concentrations were based on the minimum normal values of 6, 4 and 2 grams per 100 ml. for total protein, albumin and globulin respectively (22). The minimum normal values for the total circulating proteins in any given patient were obtained by use of these figures and the predicted plasma volume. Thus, any value for the individual total circulating proteins which exceeded the theoretical minimum normal value could be considered within normal limits for the patient.

It is apparent that the vast majority of the individual plasma volumes represent significant elevations above the predicted normal values. In 83 determinations, 68 (81.9 per cent) were more than 10 per cent above predicted values. Thirty (36.1 per cent) were more than 50 per cent above normal. In one patient (J. C.), nine of 12 determinations were more than 50 per cent above normal. Twelve determinations (14.4 per cent) were within plus-minus 10 per cent of predicted values. The three remaining determinations were more than 10 per cent below normal. Despite fluctuations, sustained elevations in plasma volume were present in most of the patients.

The total blood volume was estimated 79 times. Of these, 60 (75.9 per cent) were more than 10 per cent above predicted values. Eleven (13.9 per cent) were more than 50 per cent above normal. Of the remaining determinations, 14 (17.7 per cent) were considered to be within normal range and five (6.3 per cent) were more than 10 per cent below normal.

Total protein concentration in the serum was determined 84 times on the 12 patients. Of these, 34 (40.4 per cent) were below 6 grams per 100 ml. Three patients (A. B., C. P., E. D.), accounting for nine determinations, had total protein concen-

trations consistently above 6 grams per 100 ml. Thus, the remaining nine patients were considered to have had hypoproteinemia either consistently or at some time during the course of their study.

The quantity of total circulating protein was found to be within theoretical normal limits in 69 (86.2 per cent) of 80 determinations. Five patients (C. P., F. P., E. D., A. B., J. G.) had total circulating protein values consistently within the range of normal. These five patients accounted for 19 of the 80 determinations.

Albumin concentration in the serum was determined 82 times. Of these, 75 (91.4 per cent) were below 4 grams per 100 ml. Each patient exhibited hypoalbuminemia, as determined by concentration levels, almost consistently throughout his period of study. At no time did eight of the patients reach a normal value for albumin concentration.

The quantity of total circulating albumin was found to be within theoretical normal limits in 32 (41.0 per cent) of 78 determinations. Only one patient (J. G.) had consistently normal values. This patient accounted for six of the determinations.

Globulin concentration in the serum was determined 81 times. In only four instances was the globulin concentration below 2 grams per 100 ml. This represented 4.9 per cent of the total number of the determinations.

The quantity of total circulating globulin (77 determinations) was found to exceed the theoretical minimum normal values in every instance.

Fibrinogen concentration was determined 82 times. In 64 (78.0 per cent) the concentration was above 200 mg. per 100 ml. In 75 (94.9 per cent) of 79 determinations, the quantity of total circulating fibrinogen was found to be above the theoretical minimum normal values. One patient (J. C.), at a time when the plasma volume was 5080 ml., had a fibrinogen concentration of only 72.5 mg. per cent which represented a total circulating value of 3.6 grams. Another patient (H. R.) had moderately low values on two occasions; at one time the plasma volume was 2622 ml., the fibrinogen concentration 127 mg. per cent and the total circulating quantity of fibrinogen 3.3 grams. At another time the plasma volume was 2278 ml., the fibrinogen concentration 99.8 mg. per cent and the quantity of total circulating fibrinogen 2.2 grams. A third patient (A. D.) accounted for the

fourth subnormal value. The plasma volume was 4550 ml., the fibrinogen concentration 126 mg. per cent and the quantity of total circulating fibrinogen 5.7 grams. At no time did any of the patients exhibit hemorrhagic tendencies which could be attributed to subnormal values for fibrinogen.

From these observations it is apparent that the serum protein concentrations alone cannot be used to estimate the quantity of total circulating proteins in patients with advanced Laennec's cirrhosis of the liver. It is also apparent that any estimation of the degree of protein synthesis depends, in part, upon the determination of total circulating quantities rather than concentrations in the serum. Thus, patients with low concentrations of proteins in the plasma may, by virtue of sustained elevations in plasma volume, have total circulating quantities within the limits of normal. These data, based upon the total number of individual determinations, are summarized in Table II.

TABLE II

Tabular listing of the number of individual determinations on 12 patients with advanced Laennec's cirrhosis of the liver showing both numerical and percentile relationship between concentration of proteins and circulating proteins

	Number of determinations	Number of determinations above minimum normal value	Per cent of determinations above minimum normal value
Plasma volume	83	68*	81.9
Total protein concentration	84	50	59.6
Total circulating protein	80	69	86.2
Albumin concentration	82	7	8.6
Total circulating albumin	78	32	41.0
Globulin concentration	81	77	95.1
Total circulating globulin	77	77	100.0
Fibrinogen concentration	82	64	78.0
Total circulating fibrinogen	79	75	94.9

* Number of plasma volumes more than 10 per cent above predicted values.

Attempts to evaluate response to treatment were limited to seven patients all of whom were studied for more than 90 days. Since results of the four hepatic function tests discussed above could not be correlated with the clinical state of the patient, clinical improvement was based specifically upon disappearance of edema and ascites, return to a clinically normal nutritional state, improved or normal blood count and, finally, ability to remain ambulatory without recurrence of preexisting signs and symptoms. The maintenance of a continued state of improvement while fully ambulatory out-

side the hospital was considered an important factor in evaluating prognosis.

Only three patients (J. C., J. G., C. W.) satisfied these criteria for improvement. The first two patients have been observed for four months following discharge from the hospital. The third (C. W.) has remained as a hospital employee. A fourth patient (F. P.), in whom temporary improvement occurred, failed towards the end of the study. Coincident with this failure to maintain an improved state was the demonstration of a filling defect in the stomach. Ability to maintain a quantity of total circulating albumin above predicted minimum values occurred in three of these patients and temporarily in the fourth (F. P.) despite the presence of hypoalbuminemia as estimated by concentrations in the serum. Another patient (J. R.) showed only moderate improvement while in the hospital and has not been adequately followed since discharge. One (G. O.) has shown no improvement over a long period of time and one (T. M.) expired from his disease and complicating bronchopneumonia. These last three patients were not able to maintain a quantity of total circulating albumin above predicted minimum values.

Of the remaining five patients, all of whom were studied for less than 90 days, only two (E. D., C. P.) are making satisfactory progress. One (A. B.) left the hospital before response to treatment could be evaluated. Another (H. R.) expired in severe hepatic decompensation complicated by bronchopneumonia. The fifth (A. D.) expired following a "button" operation performed in another hospital without our knowledge. This patient had entered the hospital with a history of having had 96 abdominal paracenteses during the preceding 12 months. Routine studies revealed the presence of peritoneal tuberculosis complicating the cirrhosis. Despite this complication and the loss of large amounts of albumin in the ascitic fluid, a near normal quantity of total circulating albumin was maintained in the plasma until his demise.

Although this series is small, clinical improvement, based on criteria given above, could be correlated with ability to maintain a quantity of total circulating albumin above predicted minimum values. Since all of the patients exhibited subnormal concentrations of albumin in the serum, this correlation existed only in those who had sustained

TABLE III

Tabular listing of 12 cases of advanced Laennec's cirrhosis of the liver to illustrate the average per cent deviation from the predicted plasma volume and from the minimum normal values for both concentrations of proteins in the serum and total circulating proteins in individual patients

	Concentrations				Circulating proteins		
	P. V.	T. P.	Alb.	Glob.	T. C. P.	T. C. A.	T. C. G.
J. C.	+69.5	+7.6	-13.1	+49.2	+84.5	+50.2	+153.1
J. G.	+63.3	+12.7	-14.2	+67.1	+86.1	+41.0	+76.3
C. W.	+3.8	+14.9	-16.4	+76.6	+19.6	-14.1	+87.2
F. P.	+56.1	-2.8	-33.3	+70.6	+49.5	+3.8	+162.0
J. R.	+63.5	+0.8	-43.3	+93.3	+48.5	-23.0	+179.0
A. D.	+24.7	-9.5	-29.0	+29.3	+10.7	-13.1	+58.3
T. M.	+28.7	-14.7	-43.9	+40.9	+9.9	-26.1	+80.8
G. O.	+53.7	+0.5	-37.5	+76.7	+53.6	-3.4	+167.5
E. D.	+24.2	+17.0	-26.8	+104.7	+46.7	-9.8	+160.1
H. R.	+11.4	+8.3	-55.7	+136.5	+21.5	-49.0	+162.5
C. P.	+4.8	+15.3	-10.0	+66.3	+20.3	-7.5	+76.0
A. B.	-9.6	+16.3	-37.8	+124.7	+4.7	-45.1	+104.5

elevations in plasma volume. In most instances wide fluctuations in serum protein concentrations were not present. Three of the four patients who made the greatest degree of improvement possessed high plasma volumes and quantities of total circulating albumin above predicted minimum values throughout most of the study. The fourth patient (C. W.) eventually became able to maintain a normal quantity of total circulating albumin without an appreciable elevation in plasma volume.

Observations relative to the average percentile deviation from the predicted plasma volume and from the minimum normal values for both concentrations and total circulating proteins for each patient are presented in Table III. In most instances fluctuations in plasma volume in any given patient were paralleled by similar fluctuations in the quantity of total circulating protein. This observation is illustrated in Figure 1 which records the data on one of the patients.

DISCUSSION

The sustained elevations in plasma volume in cirrhotic patients cannot be explained by behavior of the dye. Wide variations in protein concentration do not appreciably affect the optical density of T-1824 (23). In accord with reports elsewhere (13, 24), we were unable to demonstrate the presence of the dye in the ascitic fluid as long as one hour after the intravenous injection of large amounts. On the basis that intra-abdominal pressure might act as a barrier preventing diffusion of the dye into the peritoneal cavity, the ascitic fluid was largely removed. Again, the dye failed to appear in the remaining ascitic fluid as long as one hour later under similar conditions. Under cer-

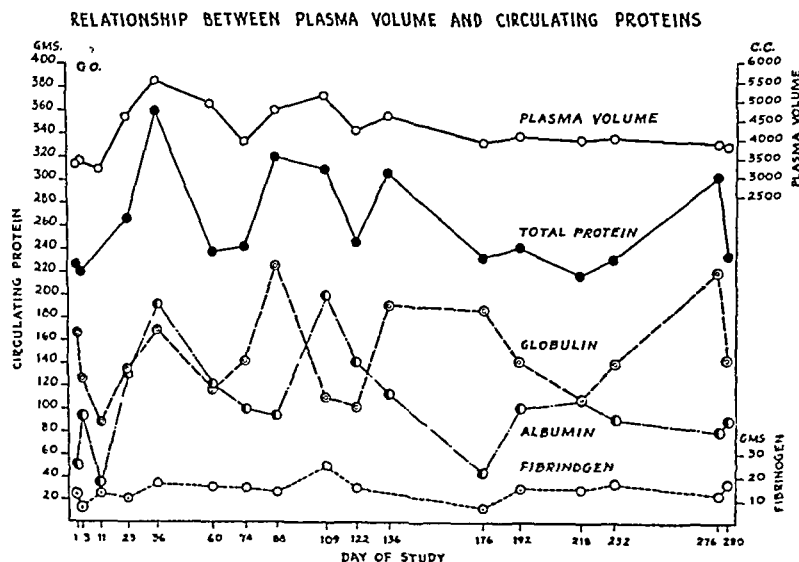


FIG. 1

Predicted normal values for patient G. O. are: Plasma volume, 2750 ml.; total circulating protein, 165 grams; total circulating albumin, 110 grams; total circulating globulin, 55 grams; total circulating fibrinogen, 5.5 grams.

tain circumstances, however, the dye may appear in the ascitic fluid. One patient developed a distinct blue color in the ascitic fluid after a large number of plasma volume determinations were made with T-1824 in a relatively short period of time. Inspection of the time-concentration curves did not reveal any sudden or unexpected loss of dye from the vascular system in any of the patients. It is undisputed that dye eventually escapes into the interstitial fluid and certainly into the ascitic fluid, but not appreciably so during the time required to perform the plasma volume determination.

Other factors in the cirrhotic patient may contribute to the elevated plasma volume. Hydremic hypovolemia in the severe stages of pernicious anemia and increases in plasma volume in the hypochromic anemias have been reported elsewhere (25). In nearly all instances the patients reported here exhibited at least moderate degrees of anemia and reduced hematocrit values at some time during the course of their study. Inability of the cirrhotic liver to normally inactivate the posterior pituitary antidiuretic factor (26) and to normally inactivate and conjugate estrogens undoubtedly contributes to the elevated plasma volume in certain instances. The relation of the normal and pathologic liver to estrogen metabolism has recently been reviewed (27). The water-retaining effect of estrogens (28) is now well known. The urine of cirrhotic patients with edema has been found to contain a potent antidiuretic factor (29). Recently, a vasodepressor substance (VDM) of hepatic origin has been shown to appear in the blood of decompensated cirrhotic patients (30). Still more recently, it has been shown that cirrhotics with edema release only very small amounts of sodium into the urine (31). Lastly, it has been suggested that the chief factors responsible for the elevated plasma volume are distension behind the point of portal obstruction and the increased size of the collateral vascular bed required to handle the portal circulation (13).

Reference to the plasma volumes in any given patient in this series reveals no significant or consistent correlation with the presence or absence of anemia, edema or ascites. In the four patients in whom edema and ascites disappeared and, clinically, the nutritional state returned to normal, the plasma volumes remained elevated. Three of

these improved patients had unusually high plasma volumes which were sustained throughout the study. Such elevations, in the absence of edema, cannot be explained on the basis of sodium retention, hypoproteinemia or disturbed estrogen metabolism. Since the plasma volume tends to increase in anemic states, anemia probably was a contributing factor in most of these patients. The presence of antidiuretic factors was not investigated but also would seem unlikely as a major cause of the elevation in the absence of edema. The patients reported here exhibited evidence of collateral circulation manifested by hemorrhoids, dilated superficial abdominal veins and esophageal varices. All had large livers and two had large spleens. Since the large liver and extensive collateral vascular bed must add a considerable increment to the capacity of the vascular system, it is suggested that a large portion of the sustained elevation in plasma volume in advanced Laennec's cirrhosis has its basis in this explanation.

Most of these patients received a mercurial diuretic and ammonium chloride at regular intervals during the first half of the study. Although small fluctuations in plasma volume occur during active diuresis, this is a temporary phenomenon and does not influence the sustained increase. Furthermore, inspection of plasma volume curves for each patient before and after diuresis revealed no significant alterations which could be attributed to the use of diuretics.

In some of the patients changes in plasma volume, at a time when hematocrits remained relatively constant, indicated tremendous increases in circulating red cell mass. In this respect it should be emphasized that determination of the circulating red cell mass by means of the plasma volume and hematocrit affords only a rough estimate. A macrocytic anemia was present at one time or another in all of the patients but not consistently so. Bone marrow studies were not obtained on these patients; however, other studies have indicated that increased erythropoiesis is frequently present in cirrhosis of the liver. Berman *et al.* (32) reports the consistent change in the bone marrow to be extension of the marrow organ so that active hematopoiesis is found in the shafts of long bones. The bone marrow is of normal or increased cellularity, with normal or increased erythrocytogenesis, regardless of the presence or absence of anemia.

Hypocellularity of the marrow was considered an unusual finding.

The clinical significance of the serum protein changes has been recently reviewed (33). Concentrations of proteins in the serum cannot, however, be used to estimate total circulating quantities in cirrhotic patients. This is emphasized by the sustained elevations in plasma volumes in the patients reported here. That the quantity of total circulating albumin may be related to prognosis is demonstrated by the findings in four of the patients who improved according to criteria already discussed. Despite low concentrations of albumin in the serum and persistently abnormal hepatic function tests, three of these patients have shown continued improvement and ability to maintain a quantity of total circulating albumin above the theoretical minimum values. The same correlation existed temporarily in a fourth patient. Two of these patients have been under observation for more than 16 months and two for more than nine months. No correlation existed between improvement and the various serum protein concentrations, total circulating protein, total circulating globulin and several hepatic function tests. Observations (34) on the amount of protein lost in the ascitic fluid due to repeated abdominal paracenteses in some of the patients suggested that synthesis of albumin 'proceeded at an unusually high rate despite low concentrations in the serum and impairment of other liver functions.

Ability to synthesize fibrinogen in normal quantities even in the presence of advanced cirrhosis is also suggested. Apparently, fibrinogen synthesis is one of the last functions to be impaired in progressive cirrhosis of the liver.

SUMMARY

1. Periodic observations of the plasma volume, blood volume, various serum protein concentrations and total circulating proteins were made on 12 patients with far advanced Laennec's cirrhosis of the liver.

2. Considerable and sustained elevation of the plasma volume was present in most of the patients. Observations on four patients, during the absence of clinical edema and ascites, suggest that the major portion of the elevation in plasma volume is due to hepatomegaly, splenomegaly and the ex-

tensive collateral circulation which develops in certain patients with cirrhosis of the liver.

3. Subnormal concentrations of proteins in the serum did not necessarily indicate subnormal quantities of total circulating proteins.

4. Clinical improvement was correlated with ability to maintain a normal quantity of total circulating albumin in four of the patients. It is suggested that this may be an important factor in determining prognosis in the cirrhotic patient.

5. It is possible for patients with advanced cirrhosis of the liver to show clinical improvement for long periods of time despite persistent impairment of liver function as determined by several hepatic function tests and despite the presence of subnormal concentrations of albumin in the serum.

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THE AGGLUTINATION OF AUTOCLAVED HEMOLYTIC STREPTOCOCCI BY SERUM FROM PATIENTS WITH RHEUMATIC FEVER AND OTHER CONDITIONS¹

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Thulin (1-3) has recently reported an agglutination test, using human serum and hemolytic streptococci which had been treated at a high temperature. He found that in human sera the agglutination titers were significantly higher in samples from patients with rheumatic fever and with rheumatoid arthritis than in others and that the titers observed in both diseases were similar. This test would seem to offer an unusually simple technique for the measurement of antibodies which occur in active streptococcal diseases and in rheumatoid arthritis. Many streptococcal agglutination tests have been described and proposed in the past, but the principle of subjecting the bacterial suspension to a temperature of 120° C prior to its use as an agglutinin (4), is a new suggestion insofar as its clinical application for the study of streptococcal diseases is concerned; for this reason alone it has seemed to deserve further investigation.

It is the purpose of this paper therefore to report confirmation of Thulin's observation that sera from patients with rheumatic fever and rheumatoid arthritis agglutinate autoclaved streptococci and to present the results of further investigations utilizing this technique.

MATERIALS AND METHODS

1) *Preparation of bacterial suspension.* The hemolytic streptococcus³ used throughout the present work was a strain of Group A Type 4. Cultures were made with beef heart infusion broth containing either 0.5% calf serum or 1% glucose; after incubation at 37° C for 15 to 18 hours, the bacterial cells were collected by centrifugation and washed once with phosphate buffer solution (0.01 M) at pH 7.8. The cells were then resuspended in the buffer and autoclaved at a temperature of

120° C for two hours. The heat-treated cells were then washed twice with the buffer (pH 7.8). Finally, a heavy suspension of the autoclaved bacteria was made in a small volume of physiological saline, buffered with 0.01 M phosphate to pH 7. Tests done with unbuffered saline prepared from laboratory-distilled water, which was found to have a pH of about 5 to 6, gave non-specific agglutination.

For the agglutination test, the bacterial suspension was diluted to a concentration of about 100 million organisms per milliliter by comparison with a barium sulphate standard, containing a third of the strength of the No. 1 tube of the Brown's standard (5, 6). The undiluted bacterial suspension was usually not kept longer than one week in the refrigerator at a temperature of 4° C. Diluted suspensions were freshly made for each test from the stock.

2) *Serum.* Samples of serum were obtained from patients suffering from rheumatic fever of varying severity and at different stages of their illness; from patients with scarlet fever or other bacteriologically proven streptococcal diseases taken during active and convalescent stages of their illness; from patients affected by rheumatoid arthritis, taken at various stages of the disease; from individuals with a wide variety of ailments other than the above three; and from "normal" subjects.⁴ All the samples of serum were stored at -17° C and were not heated before being tested.

3) *The agglutination technique.* Serial two-fold dilutions of serum were made with buffered saline (pH 7), starting from 1:20. To 0.2 ml of serum dilution in each tube an equal volume of the bacterial suspension was added. Therefore, the final dilutions of each serum tested ranged from 1:40.

After the addition of the cells to the serum, the tubes were shaken, incubated in a 52° C waterbath for two hours and left in the refrigerator at 4° C for a period of 15 to 18 hours. A longer time in the refrigerator tended to give rise to excessively high titers, while too short a period resulted in low titers. When the test was read, the individual tubes were gently shaken and then read

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² Milbank Memorial Fund Fellow.

³ The term "hemolytic streptococcus" throughout this paper refers to the Lancefield Group A beta-hemolytic streptococcus, unless stated otherwise.

⁴ The serum samples were collected and generously made available by Dr. Robert W. Quinn from the patients in the Grace-New Haven Community Hospital and Dispensary Clinics, New Haven, Conn., the U. S. Naval Hospital, St. Albans, L. I., St. Francis Sanatorium for Cardiac Children, Roselyn, L. I., the Children's Center, New Haven, Conn., and Southbury Training Center, Southbury, Conn.

against a black background, without the aid of a hand lens. The presence of sandy granules which were not broken into a homogeneous suspension by the shaking was considered as definite agglutination. The final dilution of the serum in the last tube of the series which showed definite agglutination was taken as the agglutination titer of the sample.

4) *Calculations.* In this agglutination test, two-fold dilutions of serum were used and the final dilution of

serum was adopted for the agglutination titer of the sample. This serial two-fold dilution of serum forms a geometric series with a common ratio of 1:2. To obtain a geometric mean from numericals of the dilutions is not too easy a job. If it could be converted into an arithmetic series, the task would then not be difficult. The series of dilutions of serum starts with 1:40. Here $40 = 20 \times 2^1$. The next tube contains a dilution of serum of 1:80, where $80 = 20 \times 2^2$. The third tube contains a

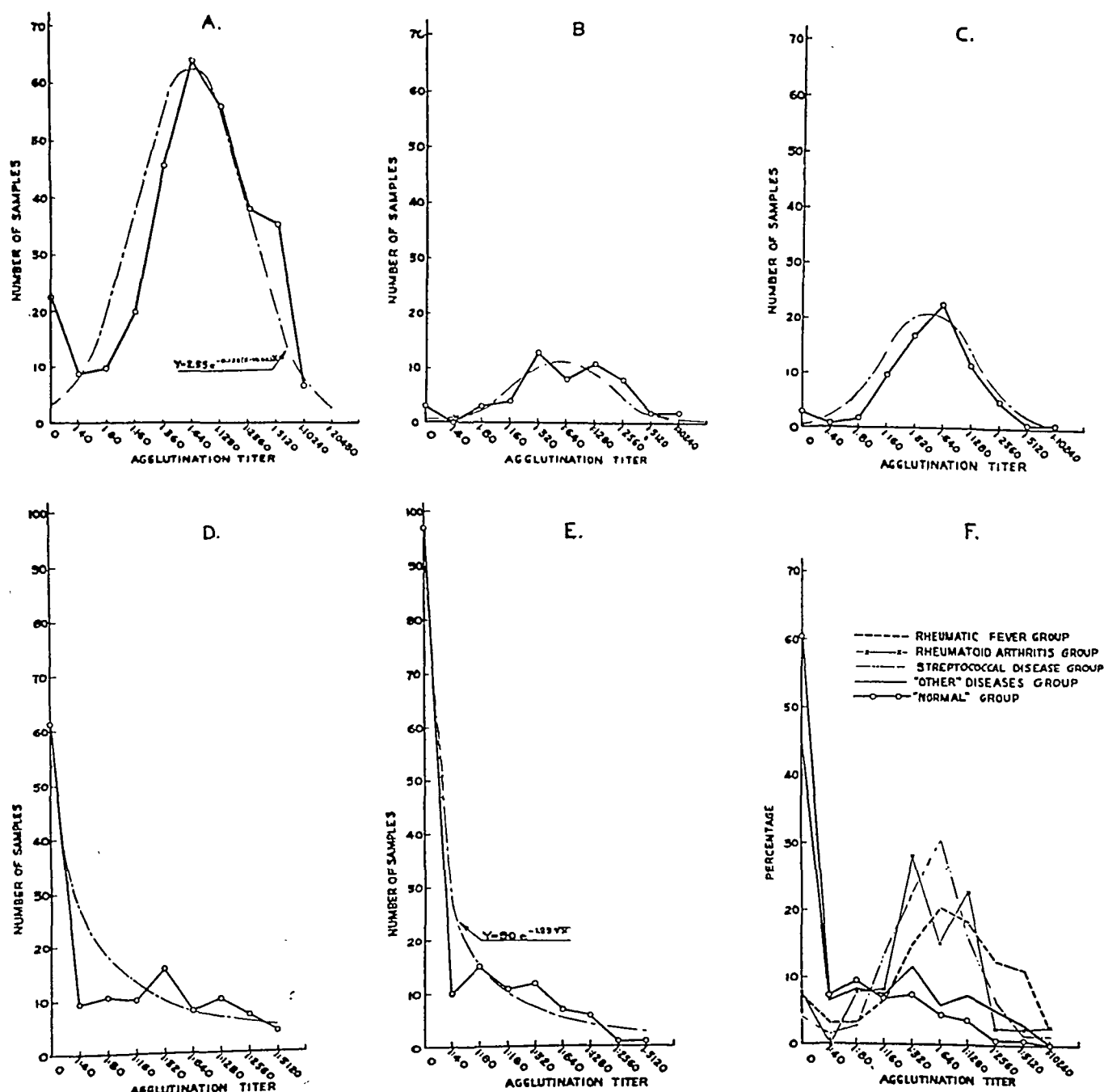


FIG. 1. DISTRIBUTION OF AGGLUTINATION TITERS OF THE FIVE GROUPS OF HUMAN SERUM

- A. Serum from patients with rheumatic fever. (311 samples, mean titer 1:646.)
 B. Serum from patients with rheumatoid arthritis. (50 samples, mean titer 1:614.)
 C. Serum from patients with "streptococcal diseases." (75 samples, mean titer 1:474.)
 D. Serum from patients with "other" diseases. (137 samples, mean titer 1:95.)
 E. Serum from "normal" subjects. (160 samples, mean titer 1:49.)
 F. Percentage distribution of the five groups.

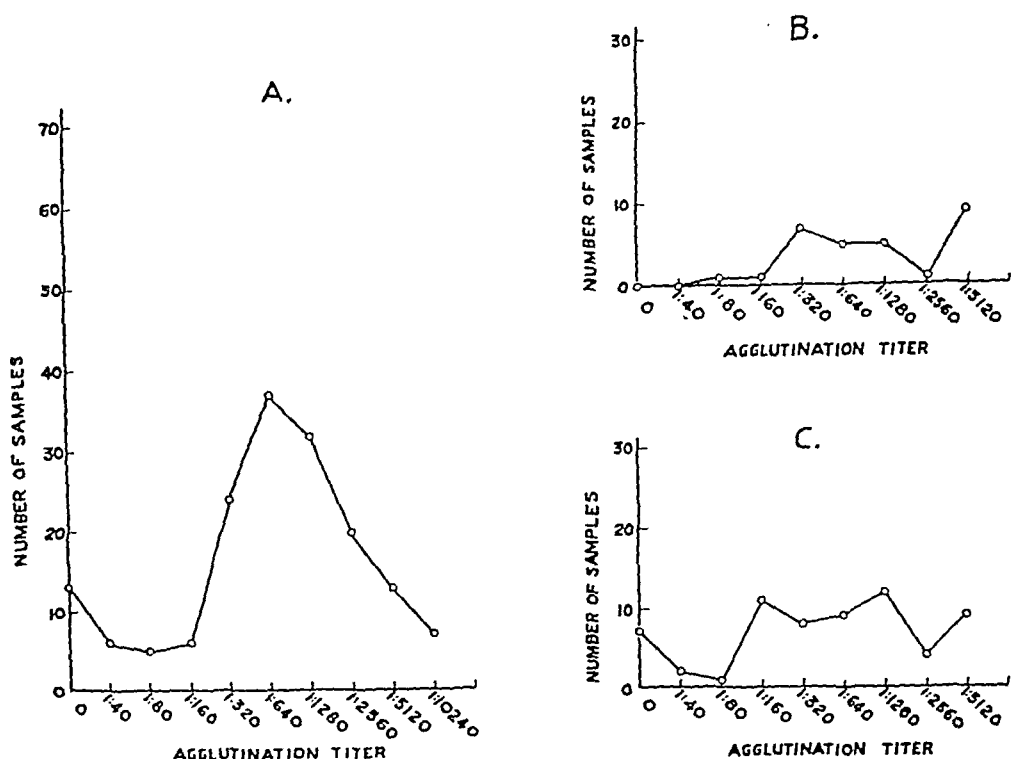


FIG. 2. FREQUENCY DISTRIBUTION OF AGGLUTINATION TITERS OF SERUM FROM RHEUMATIC FEVER PATIENTS

A. Active. (163 samples, mean titer 1:640.)

B. Active subsiding. (29 samples, mean titer 1:1084.)

C. Inactive. (63 samples, mean titer 1:456.)

dilution of 1:160, where $160 = 20 \times 2^2$. And so on. The ninth tube contains a dilution of 1:10240, where $10240 = 20 \times 2^9$. This series may, then, be coded with the first tube (1:40) as 1, second tube (1:80) as 2, . . . the ninth tube (1:10240) as 9, and with a negative reaction as 0 (Table I). Thus, the mean, the standard deviation, and the standard error of a group of serum may be calculated as usual. The *t*-test is used to compare the differences between two groups.

For converting the code of the mean into the original series, the following procedure may be taken. Taking the first group of serum as an example, the mean was calculated to be 5.01 in terms of the code. 5.01 lies between 5 and 6. 5 corresponds to $1:20 \times 2^5$. 5.01 should correspond to $1:20 \times 2^{5.01}$, or 1:645.

RESULTS

The first group consisted of 311 samples of serum obtained from 193 patients with rheumatic fever representing wide variations in severity and duration of activity of the disease. Only 23 of the 311 (7.4%) failed to show any agglutination (negative), and 269 (86.5%) showed agglutination

titers of 1:160 or higher. The geometric mean titer of the group as a whole was 1:645 (or 5.01, in terms of the code, with a standard deviation of 2.26 and standard error of 0.128) (Figure 1-A).

Sera from some of the rheumatic fever patients were further studied to determine the relation of the agglutination titer to the stage of the illness. Of the 311 samples of sera (charted in Figure 1-A), 49 were obtained from rheumatic fever patients whose detailed histories were not definitely known. Since the stage of their disease could not be determined accurately they were excluded from this analysis. One hundred and sixty-three samples were taken during the active phase of their illness and of these, 13 (8%) were negative and 139 (85.3%) had a titer of 1:160 or higher, with a mean agglutination titer of 1:640 (or 5.0, in terms of the code, with a standard deviation of 2.29 and a standard error of 0.182). It is noteworthy that the specimens of serum from ten of the 13 negative reactors were obtained within one week of the onset of their disease (Figure 2-A).

Twenty-nine samples were obtained from rheumatic fever patients when the activity of the disease was considered to be subsiding. None of these was negative and the agglutination titer of 28 samples was 1:160 or higher, with a mean titer of 1:1084 (or 5.76, in terms of the code, with a standard deviation of 3.01 and standard error of 0.558) (Figure 2-B).

Of 63 specimens of sera obtained from rheumatic fever patients during the inactive phase of their illness; seven (11.1%) were negative and 53 (84.2%) had a titer of 1:160 or higher with a mean titer of 1:456 (or 4.51, in terms of the code,

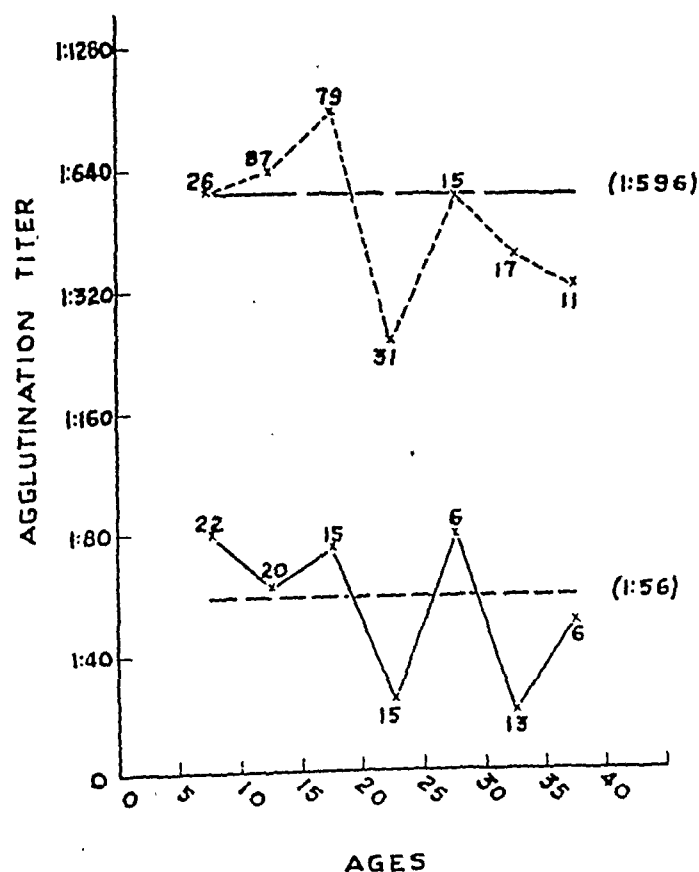


FIG. 3. COMPARISON OF MEAN AGGLUTINATION TITERS OF RHEUMATIC FEVER PATIENTS AT VARIOUS AGES WITH THOSE OF "NORMALS" OF THE SAMPLE AGE GROUP

In the top graph, the dotted line and crosses represent the distribution of mean agglutination titers of the rheumatic fever group at various ages; and the horizontal broken line, the grand mean titer of the rheumatic fever group (1:596 for a total of 266 samples).

In the lower graph, the lines and crosses represent the distribution of mean agglutination titers of the "normal" group at various ages; and the horizontal broken line, the grand mean titer of the "normal" group (1:56 for a total of 97 samples).

The numerals at each point indicate the number of samples examined at that particular age group.

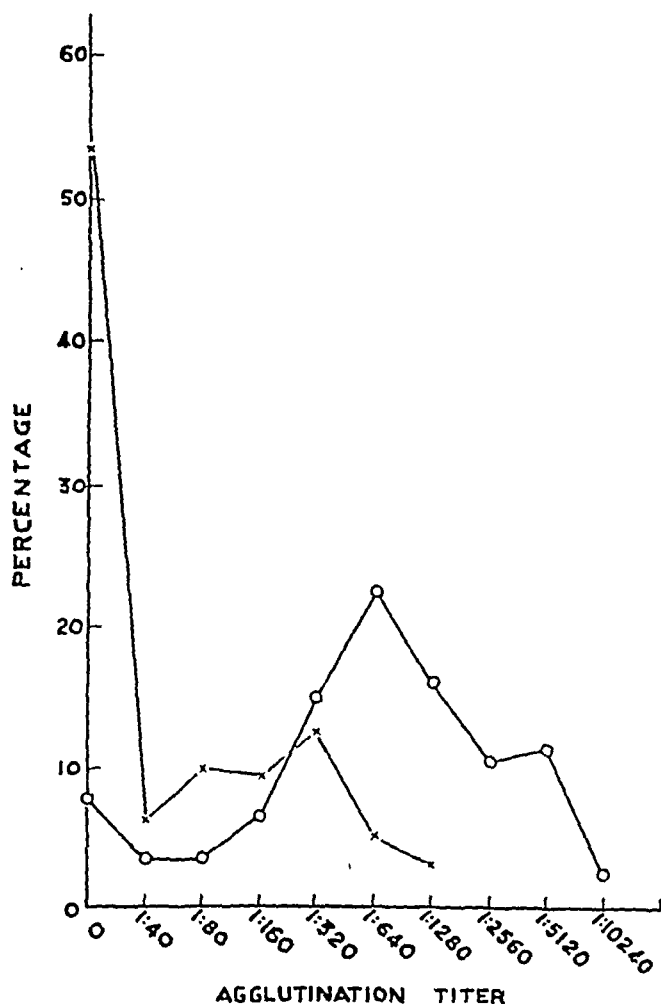


FIG. 4. PERCENTAGE DISTRIBUTION OF AGGLUTINATION TITERS OF SERUM FROM RHEUMATIC FEVER PATIENTS AND FROM "NORMAL" SUBJECTS OF AGES BETWEEN FIVE AND 40

The circles represent rheumatic fever patients; the crosses, "normal" subjects.

with a standard deviation of 2.42, and a standard error of 0.305) (Figure 2-C).

There were seven samples of serum from patients with a diagnosis of possible rheumatic fever; and of these one was negative, and six had titer above 1:160. The mean agglutination titer was 1:640 (or 5.0 in terms of the code, with a standard deviation of 1.73, and standard error of 0.655).

From our data, the ages of 268 rheumatic fever patients were definitely known. Of these, 266 fell between the ages of five and 40. The mean agglutination titers of serum samples grouped in five-year periods were computed. The mean agglutination titer of the whole group was estimated to be 1:596. Of the same age group (five to 40), 97 samples from normal controls were recorded. They were analyzed in the same manner. The

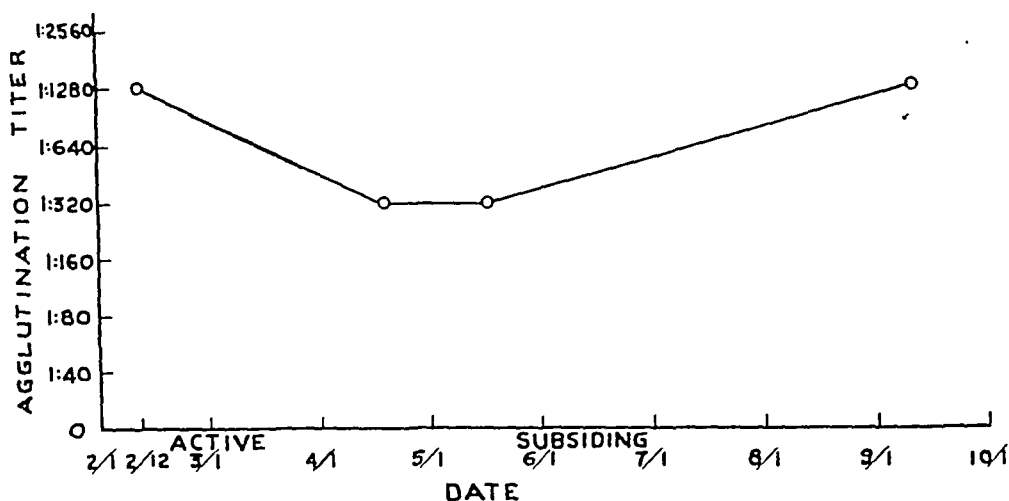
mean agglutination titer of this group was 1:56. The difference between the mean agglutination titers of these two groups of population at the same ages was statistically significant (Figure 3). The percentage distributions of agglutination titers of these two groups were essentially the same as the original corresponding group (Figure 4).

Serial bleedings of three or more samples from each individual were obtained from 32 patients of the rheumatic fever group; they represented periods of observation varying from 21 to 245 days. In general, the agglutination reaction was negative in the first few days after the onset of the acute ill-

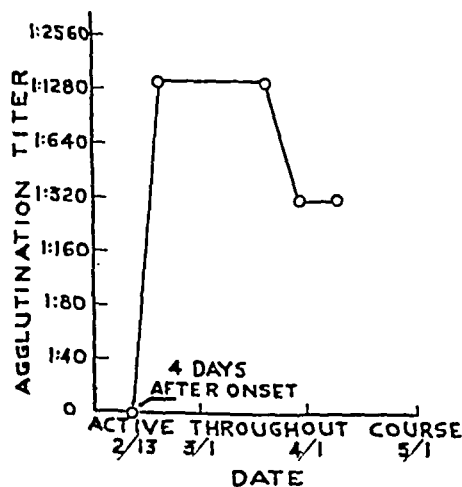
ness; thereafter the titer rose fairly rapidly to a level of 1:320 or higher during the active stage; and during the convalescent phases, it usually remained at this high level. In the few instances when the agglutination titer fell, the level, nevertheless, remained much higher than the mean titers of the control groups. Readings from three representative patients are shown in Figure 5.

Of the *second* group, comprising 50 samples of sera from 38 patients suffering from rheumatoid arthritis, three (6%) were negative and 44 (88%) had titers of 1:160 or higher. The mean titer for the group was 1:614 (or 4.76 in terms of the code,

PATIENT WW



PATIENT SE



PATIENT PC

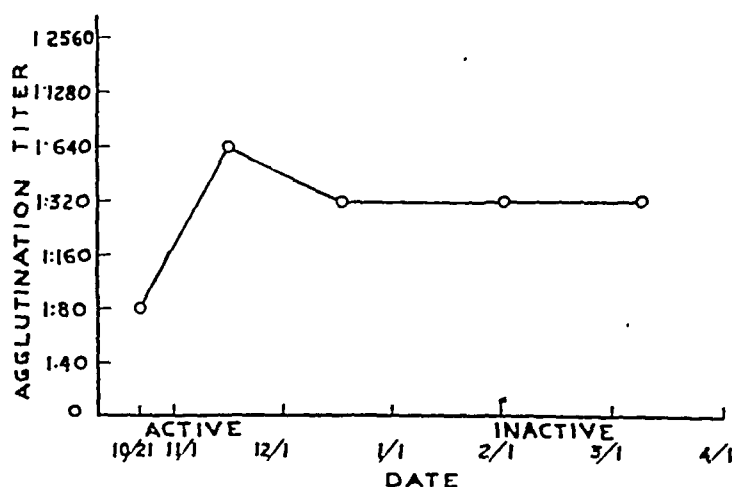


FIG. 5. THE AGGLUTINATION TITER OF SERUM FROM THREE PATIENTS WITH RHEUMATIC FEVER AS CORRELATED TO THE CLINICAL COURSE OF THE DISEASE

TABLE 1

The distribution of the agglutination titers of the five groups of serum examined

Titer	Code	Rheumatic fever		Rheumatoid arthritis		Streptococcal diseases		"Other" diseases		"Normal" subjects	
		No.	%	No.	%	No.	%	No.	%	No.	%
Negative	0	23	7.4	3	6	3	4.0	61	44.5	97	60.6
1:40	1	9	2.9	0	0	1	1.3	10	7.3	10	6.2
1:80	2	10	3.2	3	6	2	2.7	11	8.0	15	9.4
1:160	3	20	6.4	4	8	10	13.4	10	7.3	11	6.9
1:320	4	46	14.8	13	26	17	22.6	16	11.6	12	7.5
1:640	5	64	20.6	8	16	23	30.6	8	5.8	7	4.4
1:1280	6	57	18.4	11	22	12	16.0	10	7.3	6	3.8
1:2560	7	39	12.6	4	8	5	6.7	7	5.1	1	0.6
1:5120	8	36	11.5	2	4	1	1.3	4	2.9	1	0.6
1:10240	9	7	2.2	2	4	1	1.3	0	0	0	0
Total for each group		311	100.0	50	100	75	99.9	137	99.8	160	100
Mean titer for each group		1:645		1:542		1:472		1:95		1:49	

with a standard deviation of 1.68 and standard error of 0.194) (Figure 1-B).

The *third* group of sera comprised 75 samples from 65 patients who were either in the active or convalescent stage of scarlet fever or other bacteriologically proven streptococcal infections. Three (4%) of the 75 samples collected were negative and 69 (92%) showed a titer of 1:160 or over, with a mean titer of 1:472 for this group (or 4.56 in terms of the code, with a standard deviation of 1.68 and standard error of 0.194) (Figure 1-C).

The *fourth* group (one of the two control groups) of sera included 137 specimens of serum obtained from 133 patients in the Grace-New Haven Community Hospital with infectious and non-infectious diseases other than those given above. Of the 137 sera in this group, 61 (44.5%) gave negative results and 55 (40.2%) had an agglutination titer of 1:160 or higher. The mean agglutination titer was 1:95 (or 2.24 in terms of the code, with a standard deviation of 2.53 and a standard error of 0.216) (Figure 1-D).

In this fourth group, 11 patients suffering from lobar pneumonia were also included. The sera of nine of them gave negative results and two demonstrated an agglutination titer of 1:80 with a mean titer of 1:17. This fourth group also included 14 patients with tonsillitis in whom definite bacteriological proof for hemolytic streptococcus as the causative organism was lacking. Among these 14 the sera of three gave a negative reaction, while

those of ten gave agglutination titers of 1:160 or higher. The mean titer of the 14 was 1:290 (or 3.86 in terms of the code, with a standard deviation of 2.50 and standard error of 0.664). After the exclusion of these 14 patients, the mean agglutination titer of the fourth group became 1:83 (or 2.06, with a standard deviation of 2.48 and a standard error of 0.223).

The *fifth* group (the other control group) comprised sera from "normal" subjects, and included apparently healthy subjects, surgical patients such as those with simple fractures or hernias, and those admitted for routine tonsillectomy and adenoidectomy. From 142 "normal"⁵ subjects, 160 samples of sera were obtained. Of the 160, 97 (60.6%) were negative and 38 (23.8%) showed a titer of 1:160 or higher. The mean agglutination titer for the group was 1:49 (or 1.29, in terms of the code, with a standard deviation of 1.94 and standard error of 0.153) (Figure 1-E).

A comparison was made of the above five groups with regard to the percentage distributions of their agglutination titers. This is illustrated in Figure 1-F.

No special effort was made to determine the experimental error of this particular agglutination test. However, from repeated tests on 25 samples of serum (two tests on each sample at differ-

⁵ The word "normal" is used in this paper for convenience as the designation for this heterogeneous group of subjects who were apparently healthy or had no obvious infectious diseases.

ent times and with different preparations of the bacterial suspension), the mean difference of agglutination titers was found to be less than two-fold. Therefore, a four-fold difference of the agglutination titers may be considered significant.

DISCUSSION

Significance of finding. From the observations made, there was no statistically significant difference of the mean agglutination titers among the first three groups, viz., rheumatic fever, rheumatoid arthritis and streptococcal diseases. The differences between the mean titers of each of these three groups and the fourth (or patients suffering from diseases other than the above three) were highly significant statistically. Likewise, the differences between the mean titers of each of the above first three and the last group (composing "normal" subjects) also were significant. Though a statistically significant difference between the two control groups (*i.e.*, the fourth and the fifth) was demonstrated, this might be explained by the inclusion of patients suffering from tonsillitis and other similar conditions where definite bacteriological proof of causation by the hemolytic streptococcus was not obtained. The agglutination titers of the sera of these tonsillitis patients were, on the average, higher than those of the sera of other patients. The result of the fourth group as a whole might thus be influenced to some extent. However, the exclusion of these 14 patients did not significantly alter the mean agglutination titer of the fourth group. The differences between the fourth group after exclusion of these patients and the fifth group remained still significant. Among other causes, it is conceivable that an anamnestic reaction caused by the present illness on previous experiences with the streptococcus, known or unknown, might also contribute to the slightly higher mean titer of the fourth group. Therefore, this difference between the two control groups might not be taken too seriously. The frequency distribution curves of the agglutination titers of these two control groups were essentially the same (Figure 1, D and E). Those of the first three groups followed the pattern of the normal distribution curve (Figure 1).

Because the mean agglutination titers of the control groups were below 1:100, and those of the three test groups were above 1:320, it has seemed

reasonable to set the tentative line of demarcation between the control and the test groups at the level of 1:160. Therefore, if this test is to be used for clinical diagnostic purposes and when rheumatic fever is suspected, an agglutination titer of the patient's serum against autoclaved hemolytic streptococcus above 1:160 may be considered suggestive. From the estimation made by means of an empirical equation, $y = 2.55 e^{-0.128 x (x-10.02)}$ which was constructed from the data of the rheumatic fever group, the chances that the serum sample from a rheumatic fever patient would show an agglutination titer of, or over, 1:160 were 85 times out of 100. For the serum of a "normal" subject, the chances of being so were only about 20 times out of 100, as estimated from an empirical equation, $y = 90 e^{-1.25 \sqrt{x}}$ which was computed from our data. The estimate for the latter group was inevitably rather high, perhaps because the frequent occurrence of unrecognized infections of hemolytic streptococcus may give rise to an increased antibody titer. In the rheumatic fever group, a negative agglutination reaction, or a positive one with a low titer, might be explained by the fact that, at very early stages of the disease, the individual might not have elaborated enough antibodies against the "infection" to give a positive reaction with a high titer above the normal level. Moreover, there are always individual variations in the reaction to any antigenic stimulation. It is not to be expected, therefore, to observe a uniform narrow range of antibody titer. As is well known, for example, in the production of diphtheria antitoxin some horses are encountered which are refractory to diphtheria toxin or toxoid.

Rheumatic fever. Since rheumatic fever is mainly a disease of younger age groups, the question may be raised as to whether the age distribution of the rheumatic fever patients was sufficiently different from the control groups as to make a comparison impossible. As shown in Figure 3, the differences between the mean agglutination titers of the serum of these two groups of population at the same ages were statistically significant. Furthermore, the percentage distribution of agglutination titers of these two groups did not differ from their original corresponding groups. This serves as additional evidence that the agglutination titers were higher in rheumatic fever patients than in "normal" subjects.

From the studies on serial samples taken from patients at various stages of rheumatic fever, a negative reaction was observed with sera obtained within a week of the onset of the disease and the agglutination titer subsequently rose fairly rapidly and thereafter remained elevated. Thulin (3) attributed this agglutination reaction to an "O antigen" in the hemolytic streptococci. He also observed that "the O agglutinins are acute antibodies which, as a rule, make their appearance in seven to 14 days after the onset," and "in chronic cases, . . . the O agglutinins remain relatively unchanged." A negative reaction, therefore, does not exclude the presence of rheumatic fever particularly in the early stages, but a rise of the titer during the course of the disease is very suggestive. Repeated determinations at weekly intervals would appear to be indicated when the test is used as an aid in diagnosis. The degree to which sustained elevation of streptococcal agglutinins are maintained in rheumatic fever in possible contrast to the response in acute streptococcal disease is as yet unknown.

Rheumatoid arthritis. Extensive work has been done on agglutination reactions against hemolytic streptococcus with sera from patients suffering from rheumatoid arthritis. However, not many of the previous studies included numerous samples of sera from patients with rheumatic fever. The majority of them have shown higher values for rheumatoid arthritis and thus constituted a differential test between rheumatoid arthritis and rheumatic fever. The bacterial suspensions employed were either live cultures of streptococci or streptococcal suspensions killed at 56° C. Because the methods used were so different, a comparison of the data of previous studies with the results of the present work cannot easily be made. Although Clawson *et al.* (7) and Goldie (8, 9) demonstrated no difference of agglutination titers between sera from rheumatoid arthritis and rheumatic fever patients, the bacterial suspensions used by the two groups of workers were different. The size of the population examined and the stage of disease when the samples were collected are among the important factors which contribute to the variations in antibody titer, as demonstrated by different investigators. Using autoclaved hemolytic streptococci, Thulin obtained higher agglutination titers in rheumatoid arthritis and rheumatic fever patients

than in controls. In the series published in 1947 by him (2) 34 out of 43 of the "chronic polyarthritis" group showed agglutination titers of 1:160 or higher and none was negative. Of the rheumatic fever group, 13 of 16 showed agglutination titers of 1:160 or higher and none was negative; and of the control group, 114 of 117 were negative while not a single sample had a titer of 1:160 or higher. The following information was given in his latest monograph (3): 54 patients with rheumatic fever were examined by him within three to six months after onset; 51.9% of them showed an agglutination titer of 1:160 or higher. Over half of each of 20 scarlet fever patients and of 20 patients with acute tonsillitis had agglutination titers of 1:160 or higher. Of 285 patients with rheumatoid arthritis, 51.6% gave titers of 1:160 or higher. Only 7.6% of the control serum showed agglutination titers of 1:40 or higher. Our observations, as noted above, agree well to these points.

From the present study, this agglutination test could not differentiate rheumatic fever from rheumatoid arthritis. Since the number of series of bleedings at various stages of illness obtained from patients suffering from rheumatoid arthritis was not large, it is rather difficult to make definite comment on the usefulness of the test in diagnosis. This awaits the results of further investigation. Nevertheless, from our limited data, it appeared worthwhile to use this test in patients with rheumatoid arthritis as in cases of rheumatic fever.

The similarly high agglutination titers of the sera from patients with rheumatic fever and from those with hemolytic streptococcus has pointed to a possible etiological association of hemolytic streptococcus with rheumatic fever. On the other hand, one might take this finding as analogous to the Weil-Felix reaction in rickettsial diseases. This seems less likely in view of the epidemiological evidence of an association between streptococcal infections and rheumatic fever. Whether a similar interpretation can be applied to rheumatoid arthritis is beyond the scope of the present study. One may still wonder whether similarly high agglutination titers would be obtained with other autoclaved organisms, particularly other Gram positive or negative cocci. As mentioned above in the fourth group of sera examined, nine out of 11 samples of serum obtained from patients

with lobar pneumonia failed to agglutinate the autoclaved streptococci. This offered evidence for the specificity of this particular agglutination reaction. The denaturation of protein by heat has probably left the somatic polysaccharide alone among the known factors to be considered of any consequence in serving as the antigenic fraction responsible for the agglutination reaction. Further studies on the specificity of this reaction and the possible part played by the group-specific polysaccharide have been reported elsewhere (10).

SUMMARY

An agglutination reaction using autoclaved (120° C for two hours) hemolytic streptococci has been described and investigated. It has been used in this study with samples of serum from patients with rheumatic fever, rheumatoid arthritis, and streptococcal diseases, as well as with other conditions.

The agglutination titers in groups of patients with rheumatic fever, rheumatoid arthritis, and streptococcal diseases did not differ significantly one from the other. The titers in each of these conditions were significantly higher than in the control groups.

ACKNOWLEDGMENTS

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THE CLOTTING BEHAVIOR OF HUMAN "PLATELET-FREE" PLASMA: EVIDENCE FOR THE EXISTENCE OF A "PLASMA THROMBOPLASTIN"

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The precise role of platelets in the coagulation of blood has long been a matter of debate. According to the classical theory of blood coagulation, platelets are the most important source of the thromboplastic substance necessary to convert prothrombin to thrombin (1). On the other hand Lenggenger (2, 3) believes that platelets play no part in the initiation of coagulation. It is a well-known fact that patients with thrombocytopenia rarely have defective blood coagulation as measured by the usual laboratory tests. Aggeler and his associates (4) in a recent study of 404 patients found no statistically significant correlation between platelet counts and the clotting times of whole blood. While in most cases of thrombocytopenia the clotting time of whole blood is normal, there may be a diminished consumption of prothrombin during clotting (5). Quick (6) has shown that there is a decreased consumption of prothrombin during the clotting of plasma in which the platelet concentration has been reduced by centrifugation.

Studies on the function of platelets in coagulation have been hampered by the difficulty of rendering plasma to which no anticoagulant has been added platelet-free before spontaneous coagulation occurs. However, the addition of anticoagulants has not simplified the problem. Quick (7) has called attention to the acceleration of clotting produced by decalcification and subsequent recalcification, presumably as a result of physico-chemical alterations in which platelet rupture may play a major part.

Numerous studies have been undertaken using decalcified blood. Eagle (8) has shown that the clotting time of oxalated plasma upon recalcification varies inversely with the concentration of platelets. Cramer and Pringle (9) and Eagle (8) found that oxalated plasmas after Berkefeld filtration were incoagulable upon recalcification. How-

ever, in view of our present knowledge it seems quite likely that the prothrombin was removed in the process of filtration.

Brinkhous (10) studied the recalcified plasma clotting time of citrated human plasma collected in silicone-treated apparatus. After 22½ hours of centrifugation at 14,000 RPM (about 14,000 g), this plasma was incoagulable upon recalcification. Centrifugation for 165 minutes failed to produce incoagulable plasma. This difference in clotting behavior was presumed to be due to the complete removal of platelets after the prolonged centrifugation. The author concluded that the clotting of normal plasma depends on the presence of formed elements. No platelet counts were recorded. In our experience platelets can be completely removed at 14,000 g in much less time than 165 minutes. In Brinkhous' experiment the failure of the plasma to clot might well be explained by changes not related to platelets.

A number of attempts have been made to prepare platelet-free plasma without anticoagulants. Bordet and Gengou (11, 12) collected blood in paraffin-coated vessels and removed the formed elements by centrifugation. The supernatant plasma was found to clot promptly when placed in glass tubes, but the clotting time in paraffined tubes was greatly prolonged. These authors believed that initiation of coagulation was brought about by a physico-chemical change induced in platelet-free plasma by contact with glass surfaces. Whether or not their plasmas were actually platelet-free cannot be determined.

Fuchs (13, 14) on the other hand reported the preparation of a "Stable Plasma" without the use of anticoagulants by means of special venipuncture technique, paraffin-lined apparatus, low temperatures and rapidly attained high-speed centrifugation. Although the characteristics of this "Stable Plasma" were not clearly described, Fuchs stated

that it was spontaneously incoagulable in glass and paraffined tubes. Feissly (15) and Smith, Warner and Brinkhous (16) have been unable to confirm Fuchs' observations using similar techniques.

Jaques and his co-workers (17) have introduced into blood coagulation studies a method for coating laboratory apparatus with a non-wettable surface of silicone.¹ With this apparatus it is possible to obtain a fluid plasma free of platelets without the use of anticoagulants. Jaques and his associates presented experimental data to show that as the number of platelets is reduced by centrifugation the spontaneous clotting time of this native (undecalcified) plasma becomes longer. However, their lowest reported platelet counts were in the neighborhood of 2500 per cmm., a level not approaching platelet-free. These authors believe that in silicone-coated containers the initiation of coagulation is independent of platelet action.

Quick (6) using silicone-treated apparatus found a decreased consumption of prothrombin during the process of clotting when native plasmas were subjected to "high-speed" centrifugation (3500 RPM for seven minutes) as compared to "low-speed" centrifugation. This finding was attributed to the removal of all but a few of the platelets. However, no platelet counts were recorded. Quick did not obtain spontaneously incoagulable plasmas, but he believed it highly probable that with removal of all the platelets before any disintegration occurs, a spontaneously incoagulable plasma could be obtained.

Patton, Ware and Seegers (18) studied the clotting behavior of normal dog plasma by means of silicone-treated apparatus and high-speed centrifugation. They were unable regularly to obtain spontaneously incoagulable plasma, and found that platelet-free plasma clotted as promptly in silicone-treated as in glass tubes. For this reason they concluded that there was no evidence for the view that plasma contains a soluble factor which can initiate clotting independent of platelet action. In view of the high-speed centrifugation employed (22,000–23,000 RPM) it seems possible that the plasma thromboplastic protein described by Chargaff and West (19) could have been removed in these experiments.

The purpose of our studies was to determine the clotting behavior of human platelet-free plasma

prepared by the use of silicone-treated apparatus and high-speed centrifugation.

METHODS

Preparation of "platelet-free" plasma: Needles, syringes, test-tubes and pipets were treated with silicone after the method described by Jaques (17). When drawing blood, in order to avoid contamination with tissue juice, we employed a multiple syringe technique, discarding the contents of the first syringe. Thirty to 40 ml. of blood from the second syringe were carefully placed in iced silicone-treated tubes and centrifuged at 7000 RPM (about 6000 g) at 4° C for five minutes to remove the cells and most of the platelets. The upper portion of the plasma was removed with a silicone-treated pipet and re-centrifuged at 12,000 to 14,000 RPM (17,500–22,000 g) for 10 minutes. Occasionally another centrifugation at this speed was performed. Thereafter the upper portion of this plasma was removed and stored in silicone-treated tubes in an ice bath. Normal plasma obtained in this manner remains fluid for at least several days at 4° C.

Clotting times: All clotting times were performed by a modified Lee-White three tube method at 37° C using scrupulously clean glass or silicone-treated tubes. With this technique normal individuals occasionally have a whole blood clotting time in glass tubes as long as 30 minutes, and the clotting time in silicone tubes may be much longer. For the sake of brevity only the clotting time in the third and most significant tube is recorded, although it is ordinarily wise to consider the results in all three tubes. Clotting tubes were observed for a minimum period of 24 hours.

Prothrombin times: Prothrombin times were determined by the one-stage method of Quick using a highly standardized rabbit-brain thromboplastin prepared according to the technique of Brambel (20). Since the native "platelet-free" plasma had not been decalcified, in the prothrombin estimations on this plasma 0.1 ml. of physiological saline was used in place of 0.1 ml. of M/40 calcium chloride.

Anticoagulant assays: Tests for the presence of circulating anticoagulants were performed by adding freshly drawn normal blood to the subject's "platelet-free" plasma, and noting the effect on the clotting time of the former. Details on the use of this method and its sensitivity to heparin have been reported (21).

Platelet counts: Platelet counts on whole blood were performed by a routine method employing Rees-Ecker diluent. Normal values in our laboratory are in excess of 200,000 per cmm. Platelet counts on "platelet-free" plasma were performed by introducing the undiluted plasma into a Spencer "Bright-Line" counting chamber. The use of silicone-treated cover-slips delayed clotting 30 minutes or more, enabling one to complete accurate counts. By using the fine adjustment of the microscope the entire area between the cover-slip and chamber was visualized so that platelets which had not settled to the counting chamber surface could be counted. All refractile bodies resembling platelets in any manner whatsoever

¹ General Electric Dri-Film 9987.

were counted, so that reported values are maximal and correct values are probably somewhat lower.

Preparation of plasmas with varying platelet concentrations: In order to study further the role of platelets in clotting, different specimens of the same native plasma were rendered "platelet-free" by high-speed centrifugation and platelet-rich by very low speed centrifugation. Platelet counts were performed, and the two plasmas then mixed in varying proportions.

RESULTS

Eighty-six human subjects were studied. Of these, 41 were normal individuals and the remainder were patients with various diseases.

NORMALS

Studies of the clotting behavior of normal "platelet-free" plasma correlated with the whole

TABLE I

The clotting behavior of normal "platelet-free" and "platelet-poor" plasmas correlated with the whole blood clotting times and prothrombin times of oxalated plasmas

Subject	Whole blood clotting times in glass tubes	Prothrombin times of oxalated plasma	Native "platelet-free" plasma			
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
	min.	sec.	per cmm.	min.	min.	sec.
1	11	—	60	13	—	—
2	10	—	60	15	—	—
3	16	17	20	77	—	—
			20	31	—	—
4	14	17	20	29	—	—
5	21	15	20	74	—	—
6	15	15	>1000	14	—	—
7	17	16	20	12	—	—
8	24	16	96	35	—	15
9	29	17	55	42	—	16
10	23	18	500	53	—	14
11	27	16	230	27	—	13
12	10	—	None	9	—	14
13	14	17	None	17	—	15
14	15	15	40	34	—	11
15	18	—	200	24	—	13
16	23	18	12	20	—	16
17	17	21	8	—	23	18
18	24	—	3	—	45	—
19	17	—	>1000	24	34	—
20	14	—	None	19	60	—
			9	24	34	—
21	12	—	4	31	75	—
			None	36	126	—
22	24	22	None	48	183	20
			None	41	75	23
23	11	18	None	14	16	19
24	14	19	3	35	46	19
25	15	18	50	15	15	17
26	19	—	21	8	10	—
27	32	19	11	140	>210	17
28	19	19	8	26	28	19
29	17	22	6	47	>70	19
30	19	—	16	14	30*	18
31	16	19	2	26	47*	20
32	22	21	11	62	320*	—
33	22	21	10	39	No clots 72 hrs.*	18
34	21	—	13	60	>130*	23
35	17	21	1	42	140*	20
36	15	21	1	17	No clots 48 hrs.*	19
37	17	20	3	54	No clots 72 hrs.*	21
38	19	18	5	47	165*	19
39	18	18	1	70	No clots 24 hrs.*	23
40	16	16	3	27	63*	18
41	22	16	4	33	Partial clot 24 hrs.*	—

Note: All determinations on each subject were made on a specimen obtained from the same venipuncture. All procedures were carried out at 37° C.

* In these experiments the improved technique of re-coating the silicone-treated tubes before each use was employed.

blood clotting time in glass tubes and the prothrombin time of oxalated plasma are shown in Table I.

We abandoned the routine use of silicone-treated tubes for determination of the whole blood clotting time, since the results were too variable, poor clots were formed, and it was difficult to recognize end-points. The clotting time of whole blood in silicone-treated tubes seems to be more a measure of imperfection in technique than of the inherent properties of the blood. Theoretically blood should not clot in silicone-treated tubes if technique were perfect.

It was not always possible to obtain a plasma entirely free of refractile bodies even when repeated high-speed centrifugations were used. It seemed unlikely that all of these refractile bodies were platelets, although they must be presumed to be and were recorded as such. In some instances platelets appeared to be completely absent. To emphasize the extremely small number of platelets remaining after centrifugation we have called these plasmas "platelet-free" in contrast to the "deplateletized" plasmas of Tocantins (22) which contained as many as 20,000 platelets per cmm. Furthermore, we found no difference in behavior of plasmas containing 0 to 100 platelets per cmm.

Normal "platelet-free" plasmas invariably clotted in glass tubes at 37° C in a relatively short time. There was considerable variation in the time required for this clotting. In most instances, however, the clotting time of normal "platelet-free" plasma in glass tubes was longer than that of the whole blood from which it was derived. Even with careful technique it was rarely possible to demonstrate a perfect correlation between platelet counts and clotting times (Table II). With

platelet counts under 1000 per cmm. there appeared to be no correlation between platelets and clotting time. Completely platelet-free plasmas did not have clotting times longer than plasmas containing a few platelets.

Clotting of normal platelet-free plasma in glass tubes could be prevented by the addition of sodium oxalate. This indicates that thrombin did not exist in the platelet-free plasma prior to its introduction into glass tubes. As a matter of fact, no thrombin was demonstrable until immediately prior to the onset of visible clotting.

In our early experiments we did not recoat the silicone-treated clotting tubes before each use. Under these conditions the clotting times of "platelet-free" plasmas in silicone-treated tubes were not always significantly longer than the clotting times in glass tubes. When freshly prepared silicone-treated tubes were used, a significant difference was constantly observed. In fact, in four cases (cases Nos. 33, 36, 37, 39) *the plasmas formed no clots in silicone-treated tubes at 37° C.* In two of these there was a very slight precipitation of some amorphous material but no true clot formation. In the other two cases, although the plasmas became slightly cloudy after several hours, there was not the slightest evidence of precipitation or fibrin formation. It seems possible that with constantly perfect technique one could regularly obtain platelet-free plasmas spontaneously incoagulable in silicone-treated tubes at 37° C.

When thromboplastin was added to these "platelet-free" plasmas, prompt clotting occurred in glass or silicone-treated tubes. The prothrombin times of these plasmas were almost the same as the prothrombin times of oxalated plasmas obtained from the same venipuncture. Native "platelet-free" plasmas maintained their prothrombin activity as measured by the one-stage method when they were stored in a frozen state, whereas the prothrombin times of frozen oxalated plasmas became a few seconds longer. Freezing and thawing of "platelet-free" plasma in silicone-treated tubes resulted in shortened clotting times.

The mechanism of clotting of "platelet-free" plasma was observed in Spencer "Bright-Line" counting chambers. Clotting occurred first on the cover-slip and not until later on the silvered counting chamber surface. Clotting definitely did not

TABLE II

The relationship of the spontaneous clotting times of native (undecalcified) plasma to the number of platelets present

Platelets (per cmm.)	6	28,000	56,000	140,000	280,000	420,000	560,000
Clotting time in glass tubes at 37° C. (in minutes)	48	33	24	19	15	9	11

Note: Normal native "platelet-free" and "platelet-rich" plasmas were obtained from pooled sources by high- and low-speed centrifugations respectively. Platelet counts were performed, and the plasmas mixed in varying proportions.

begin around platelets when they were present. As clotting occurred small refractile structures appeared at the crossings of the fibers. Although no platelets may have been present before clotting occurred, thousands of these refractile structures could be seen at the fiber crossings once clotting began. If a few platelets were present, these were seen to settle to the chamber surface and disappear only after clotting was well under way.

By altering the platelet concentrations of plasmas we were able to demonstrate that the degree and speed of clot retraction varied directly with the number of platelets present. This correlation was poor and irregular in glass tubes but excellent in silicone-treated tubes. Clot retraction occurred promptly with platelet-counts as low as 20,000 per cmm. in silicone-treated but not in glass tubes. The clots of platelet-free plasmas did not retract either in glass or in silicone-treated tubes.

The dynamics of the coagulation of platelet-free plasma in glass tubes was studied by determining the time of thrombin appearance, fibrinogen utilization, and prothrombin consumption. Results of a representative experiment are shown in Table III. In general there was minimal prothrombin

consumption even over a period of several hours.

In order to investigate a possible quantitative relationship between platelet concentration and the amount of prothrombin converted in the process of clotting, prothrombin times of the residual serum after clotting were determined using plasmas containing varying numbers of platelets. The results in Table IV show a striking correlation even at the higher platelet levels when glass tubes were used. In silicone-treated tubes prothrombin consumption was minimal regardless of the number of platelets present.

High-speed centrifugation did not prolong the prothrombin time of native plasma when rabbit brain extract was used as a source of thromboplastin. However, prothrombin times with Russell's viper venom² were greatly prolonged by high-speed centrifugation. Similar observations on oxalated plasmas were reported by Macfarlane (23).

Plasmas from fasting individuals gave the best results in our experiments. In grossly fatty plas-

² Stypven, Wellcome Physiological Research Laboratories, Beckenham, England.

TABLE III

The dynamics of the coagulation of normal platelet-free plasma: tests for the rate of thrombin formation, fibrinogen utilization, and prothrombin consumption

Time after plasma transferred to glass tube at 37° C.	Gross appearance of plasma	Assay for thrombin Clotting time of the mixture: 0.2 ml. oxalated plasma* 0.2 ml. platelet-free plasma	Assay for fibrinogen Clotting time of the mixture: 0.2 ml. thrombin† solution 0.2 ml. platelet-free plasma	Assay for prothrombin Clotting time of the mixture: 0.1 ml. thromboplastin 0.1 ml. prothrombin-free plasma‡ 0.1 ml. platelet-free plasma
min.		sec.	sec.	sec.
0	Fluid	No clots	7	16
1	Fluid	No clots	—	—
5	Fluid	No clots	—	—
10	Fluid	No clots	—	—
13	Small fiber	No clots	—	—
15	Definite fibrin formation	420	10	—
17	Solid clot	240	7	—
20	(Clot compressed to remove "plasma")	75	11	—
23	Re-appearance of solid clot	60	15	—
24	—	—	—	16
28	—	74 †	No clots	—
31	—	85	No clots	—
35	—	120	No clots	17
45	—	No clots	No clots	—
55	—	No clots	No clots	15
75	—	No clots	—	15
135	—	—	—	16

* Eight parts prothrombin-free plasma (barium sulfate adsorption) and 2 parts M/10 sodium oxalate.

† Thrombin, Upjohn Company, Kalamazoo, Michigan.

‡ Three parts prothrombin-free plasma (barium sulfate adsorption) and one part of M/40 calcium chloride.

TABLE IV

The relationship of prothrombin consumption in the clotting of native plasma in glass tubes to the number of platelets present, as measured by the determination of prothrombin times on serum expressed from the clots four hours after clotting

Platelets (per cmm.)	6	175,000	262,500	350,000
Clotting times in glass tubes at 37° C. (min.)	14	10	7	10
Prothrombin time of expressed serum 4 hours after clotting (sec.)	20	63	80	141

The plasmas with various platelet concentrations were prepared by mixing platelet-free and platelet-rich plasmas from the same source.

The prothrombin times were determined by adding 0.1 cc. of the expressed serum to a mixture of 0.1cc. thromboplastin and 0.1 cc. prothrombin-free plasma at 37° C. and measuring the time required for clotting.

mas coagulation often occurred in the lipid layer at the top of the tube after centrifugation.

Hemophilia

Seven patients with hemophilia were studied. Results are shown in Table V. In every instance the "platelet-free" plasma was spontaneously incoagulable in glass and silicone-treated tubes at 37° C regardless of the length of the whole blood

clotting time. No trace of fibrin was noted in any of the tubes even after a period of days or weeks. Yet the addition of rabbit brain thromboplastin produced prompt clotting, with prothrombin times comparable to those of oxalated plasmas. When hemophilic blood was centrifuged in lusteroid tubes untreated with silicone, the resultant platelet-free plasma clotted. The silicone surface therefore appeared to be necessary for the preparation of spontaneously incoagulable hemophilic platelet-free plasma. When whole blood clotting times on hemophilic blood were performed in silicone-treated tubes, the clotting times were tremendously prolonged and in some instances no clotting occurred regardless of the clotting time in glass tubes.

One untreated hemophilic patient (Case No. 42) had a normal whole blood clotting time ranging from 12 to 25 minutes during an active bleeding episode. A platelet-rich plasma obtained by low-speed centrifugation had approximately the same clotting time. However, the "platelet-free" plasma was spontaneously incoagulable in glass and silicone tubes. Thus it appears that platelets are necessary for the clotting of hemophilic plasma, although our present results are compatible with the

TABLE V

The clotting behavior of hemophilic "platelet-free" plasma

Patient	Whole blood clotting times in glass tubes	Prothrombin times of oxalated plasma	Native "platelet-free" plasma			
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
	min.	sec.	per cmm.	min.	min.	sec.
42	25	—	None	No clots	—	14
	16	—	> 1000	No clots	—	—
43*	225	15	60	No clots	—	—
	390	21	20	No clots	—	—
	300	21	20	No clots	—	15
44	325	19	60	No clots	—	19
45	600	21	1700	No clots	—	20
			None	No clots	—	20
46	33	19	290	No clots	—	17
	46	22	30	No clots	—	—
	74	—	18	—	No clots	—
47	240	—	None	No clots	No clots	—
			None	No clots	No clots	18
48	212	21	None	No clots	No clots	22
	180	23	70	No clots	No clots	24

* This patient had a positive test for the presence of circulating anticoagulant.

view that they are not necessary for the initiation of coagulation of normal plasma.

The hemophilic "platelet-free" plasmas were tested for antithromboplastic properties by determining prothrombin times with progressively diluted thromboplastin solutions. For this we used silicone-treated tubes and native "platelet-free" plasma. The results shown in Table VI reveal no definite evidence for the presence of antithromboplastic activity in hemophilic plasma. In one experiment the native plasmas were first oxalated, then incubated several minutes with thromboplastin solutions and finally recalcified. Results were the same in this experiment. Variations in the clotting times obtained with highly diluted thromboplastin solutions must be interpreted with great caution since under such circumstances it is technically difficult to reduplicate results.

Heparinized blood

"Platelet-free" plasmas were obtained from normal individuals after the intravenous injection of small quantities of heparin. In other experiments heparin in small amounts was added *in vitro* to normal "platelet-free" plasma. Results shown in Table VII demonstrate that even after minute doses of heparin, so small that the whole blood clotting times were not affected, the "platelet-free" plasmas would not clot in glass or silicone-

treated tubes at 37° C. The *in vitro* addition of minute amounts of heparin likewise resulted in the production of incoagulable plasma. The authors (24) have previously shown that there is an inverse relationship between the action of heparin and platelet concentration. With low concentrations of heparin, prothrombin determinations on the "platelet-free" plasmas gave normal results.

Dicumarolized patients

Five patients with hypoprothrombinemia due to dicumarol were studied. Results are shown in Table VIII. While the findings are somewhat variable, the "platelet-free" plasmas of these patients were often spontaneously incoagulable in glass or silicone-treated tubes at 37° C. On two occasions these plasmas gave suggestively positive tests for the presence of circulating anticoagulant. The addition of solutions of thrombin to oxalated plasmas of dicumarolized patients resulted in longer clotting times than the addition of the same thrombin solution to normal plasmas. Further studies are in progress regarding this finding.

Thrombocytopenia

Studies were performed on 12 patients with hemorrhagic diathesis associated with thrombo-

TABLE VI

Tests for the presence of antithromboplastic activity in native "platelet-free" hemophilic plasma
Prothrombin times were determined using serial dilutions of thromboplastin.

	Prothrombin times (in seconds) using serial dilutions of thromboplastin										
	Undiluted	1:10	1:50	1:100	1:200	1:400	1:600	1:800	1:1600	1:3200	Saline
Patient No. 43*	15	21	65	170	307	416	—	—	—	—	—
Control	15	19	56	108	240	320	—	—	—	—	—
Patient No. 48	16	24	—	49	65	109	—	260	345	—	>24 hrs.
Control	16	21	—	40	57	135	—	151	205	—	660
Patient No. 46	19	22	35	50	62	83	93	133	—	—	—
Control	17	21	32	44	59	77	91	112	—	—	—
Patient No. 46	17	20	29	36	51	75	—	—	—	—	—
Control	15	19	30	37	50	—	—	—	—	—	—
Patient No. 46†	22	22	32	42	64	73	96	116	165	290	—
Control	20	22	33	44	60	64	78	94	161	325	—

* This patient had a positive test for circulating anticoagulant.

† In this experiment the native "platelet-free" plasma was first oxalated by the addition of 10% by volume of M/10 sodium oxalate. 0.1 ml. of this plasma was added to 0.1 ml. of thromboplastin. After several minutes of incubation at 37° C., this mixture was recalcified by the addition of 0.1 ml. of M/40 calcium chloride solution.

Note: All tests performed at 37° C.

TABLE VII

The clotting behavior of "platelet-free" plasmas obtained from normal subjects after the intravenous injection of varying amounts of heparin as indicated

Subject		Whole blood clotting times in glass tubes	Prothrombin time of oxalated plasma	Native "platelet-free" plasma			
				Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
		min.	sec.	per cmm.	min.	min.	sec.
49	41 minutes after intravenous injection of 50 mgms. of heparin	102	—	230	No clots	—	19
50	40 minutes after intravenous injection of 15 mgms. of heparin	105	16	None	No clots	—	15
51	30 minutes after intravenous injection of 10 mgms. of heparin	27	21	36	No clots	No clots	18
52	16 minutes after intravenous injection of 10 mgms. of heparin	31	18	3	No clots	No clots	19
53	12 minutes after intravenous injection of 7.5 mgms. of heparin	19	17	3	No clots	No clots	24
54	18 minutes after intravenous injection of 5 mgms. of heparin	30	19	7	No clots	No clots	18
55	12 minutes after intravenous injection of 5 mgms. of heparin	17	21	2	No clots	No clots	19
56	16 minutes after intravenous injection of 3 mgms. of heparin	20	18	24	No clots	No clots	16
57	16 minutes after intravenous injection of 3 mgms. of heparin	17	22	16	No clots	No clots	19
58	<i>In vitro</i> dilutions of "platelet-free" plasma to: 0.01 mgms. heparin/cc. 0.001 mgm. heparin/cc. 0.0005 mgm. heparin/cc.	— — —	— — —	5 5 5	No clots No clots No clots	No clots No clots No clots	18 — —

Note: In the case of Subject No. 58 the "platelet-free" plasma was obtained first, and then varying amounts of heparin added *in vitro* to the final concentrations indicated.

cytopenia (Table IX). Three of these patients had marked prolongation of their whole blood clotting times and in each of these instances the "platelet-free" plasmas were spontaneously incoagulable in glass and silicone-treated tubes. Eight patients with essentially normal whole blood clotting times had "platelet-free" plasmas which clotted in glass tubes in a perfectly normal fashion. In all of our cases of thrombocytopenia a sensitive test for circulating anticoagulant was negative. In order to eliminate the possibility that failure of

the three "platelet-free" plasmas to clot in glass tubes could be attributed to the presence of anticoagulant in quantities too small to be detected by our routine anticoagulant assay, a further study was carried out in one case. Spontaneously incoagulable "platelet-free" plasma from patient No. 73 was added to normal "platelet-free" plasma in glass tubes. Five tenths ml. of the patient's plasma failed to prevent coagulation of 1.0 ml. of normal "platelet-free" plasma, conclusively demonstrating the absence of anticoagulant.

TABLE VIII

The clotting behavior of "platelet-free" plasma from patients with hypoprothrombinemia due to dicumarol

Patient	Whole blood clotting time in glass tubes	Pro-thrombin time of oxalated plasma	"Platelet-free plasma"			
			Platelets	Glass tube clotting time	Silicone tube clotting time	Pro-thrombin time
	(min.)	(sec.)	(per cmm.)	(min.)	(min.)	(sec.)
59	32	40	5	40	45	44
			1	44	No clots	45
60	20	107	10	<800	<800	128
61	41	52	2	No clots	No clots	51
			1	No clots	No clots	52
	26	53	11	No clots	No clots	44
			2	No clots	No clots	43
	18	34	60	35	No clots	42
62	55	27	None	No clots	No clots	24
	15	23	4	No clots	No clots	23
63	55	117	8	No clots	No clots	109

All of the determinations were made at 37° C.

Miscellaneous patients

Table IX shows the results of studies on a group of patients with hemorrhagic diathesis associated with a variety of conditions. Detailed studies on the two patients with "undiagnosed disease due to circulating anticoagulant" have already been reported (25).

The patients with liver disease were of great interest. The two (Cases Nos. 81, 82) whose "platelet-free" plasma did not clot had positive tests for circulating anticoagulant as well as a severe hypoprothrombinemia. Furthermore, the clotting times of their plasmas after the addition of a thrombin solution were tremendously prolonged as compared to the clotting times of normal plasmas, although the fibrinogen concentrations were adequate. We therefore believe that these patients had circulating antithrombic anticoagulants. The rate of fibrinolysis of the sterile recalcified plasma clot was determined in these two cases by Dr. Oscar Ratnoff. The clot lysis time of patient No. 81 was normal, but that of patient No. 82 was shortened. There was no evidence that the coagulation defect was caused by increased proteolytic activity of the serum. Many patients with more markedly increased proteolytic activity of the serum showed no abnormality of blood coagulation.

In view of the reported occurrence of circulating heparin-like anticoagulant after treatment of Hodg-

kin's disease with nitrogen mustards³ (26), three such patients were studied. None of these patients developed hemorrhagic diathesis and tests for circulating anticoagulant were negative. Their "platelet-free" plasmas were similar to those of normal individuals (Table X).

DISCUSSION

The use of silicone-treated apparatus has made possible the satisfactory preparation of a "platelet-free" plasma without the use of anticoagulants. Employing this technique we have been able to study the clotting behavior of human plasma. The most significant finding has been that *normal "platelet-free" plasma clots in a relatively short time in glass tubes at 37° C, but its clotting time in silicone-treated tubes is markedly prolonged and sometimes there is no clotting at all.* This observation suggests that contact with glass activates some plasma constituent which can initiate clotting. This factor is apparently activated slowly or not at all by contact with silicone-treated surfaces. We believe that with constantly perfect technique, platelet-free plasma would regularly be spontaneously incoagulable in silicone-treated tubes.

We have no information concerning the origin and nature of this plasma factor. It is quite possible that during the manipulations involved in our experiments a few platelets may have been broken up. However, if active thromboplastic substance were liberated in this manner, it seems reasonable to assume that the clotting time of platelet-free plasma would be the same in silicone-treated tubes as in glass tubes. There is no evidence that a silicone surface itself interferes with the clotting process, for on addition of highly diluted thromboplastin to platelet-free plasma, clotting occurs as promptly in silicone-treated tubes as in glass tubes. We are therefore unable to escape the conclusion that an inactive thromboplastin precursor in plasma is activated on contact with glass surfaces.

The existence of a plasma thromboplastin independent of platelets has been postulated by some workers (2, 3). Howell (27) believed that a continuous destruction of platelets *in vivo* led to the

³ Methyl-bis-(β -chloroethyl) amine hydrochloride, Merck & Co., Inc.

TABLE IX

The clotting behavior of "platelet-free" plasma from patients suffering from hemorrhagic diathesis due to a variety of conditions

	Subject	Whole blood platelets (per cmm.)	Whole blood clotting times in glass tubes (min.)	Prothrombin time of oxalated plasma (sec.)	Native "platelet-free" plasma			
					Platelets (per cmm.)	Glass tube clotting times (min.)	Silicone tube clotting times (min.)	Prothrombin times (sec.)
I. Hemorrhagic diathesis with thrombocytopenia								
Acute leukemia	64	18,000	36 hrs.	—	None	No clots	No clots	20
	65	44,000	19	22	9	27	32	20
		22,000	9	25	None	11	22	23
	66	12,000	88	22	None	No clots	No clots	21
	67	26,000	7	23	3	18	160	26
	68	22,000	27	23	2	180	14 hrs.	24
Idiopathic thrombocytopenic purpura	69	30,000	15	21	1	27	63	21
	70	40,000	8	18	6	15	23	17
	71	20,000	13	21	2	24	50	23
	72	32,000	31	20	4	24	37	21
Refractory anemia with thrombocytopenia	73	36,000	93	24	13	No clots	No clots	23
	74	90,000	25	21	11	90	No clots	18
					1	90	18 hrs.	18
Marchiafava-Micheli syndrome	75	68,000	29	—	3	No clots	No clots	18
II. Hemorrhagic diathesis without thrombocytopenia								
Non-thrombocytopenic purpura	76	200,000	14	18	5	11	11	18
Atypical hemorrhagic diathesis	77	226,000	21	21	13	60	No clots	22
Multiple myeloma	78	—	43	26	None	26	24	—
Undiagnosed disease with circulating anticoagulant	79†	—	120	30	None	No clots	No clots	24
	80†	—	68	20	—	No clots	No clots	15
III. Hypoprothrombinemia due to fatal liver disease								
	81†	—	109	54 (10%)*	10	No clots	No clots	54
	82†	—	24 hrs.	87 (5%)*	1000	No clots	No clots	—
	83	—	22	187 (<5%)*	45	55	78	155
					7	60	130	—

* Estimated percentage of normal prothrombin.

† These patients had positive assays for circulating anticoagulant.

Note: Patient No. 83 was not suffering from hemorrhagic diathesis, but is included for comparison with patients Nos. 81 and 82.

formation of a plasma thromboplastin, as was previously suggested by Morawitz (1). Lozner and Taylor (28) maintain that the effect of foreign surfaces such as glass is not due to lysis of platelets but rather to activation of a plasma thromboplastin. Owren (29) has presented data to refute Lenggenhager's (2, 3) conclusions concerning the existence of a plasma thromboplastic factor and considers that no substantial evidence has been

presented. On the other hand, Chargaff and West (19) by ultracentrifugation of platelet-free plasma have obtained a sediment with potent thromboplastic properties. Quick (6) and Brinkhous (10) using techniques similar to those which we have employed have concluded that normal plasma contains a soluble factor whose interaction with platelets is necessary for normal blood coagulation. However, these authors do not believe

TABLE X

The clotting behavior of "platelet-free" plasma from patients with Hodgkin's disease treated with nitrogen mustards

Subject	Whole blood clotting times in glass tubes	Prothrombin time of oxalated plasma	Native "platelet-free" plasma				Time of determination in relation to treatment with nitrogen mustards*
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times	
	<i>min.</i>	<i>sec.</i>	<i>per cmm.</i>	<i>min.</i>	<i>min.</i>	<i>sec.</i>	
84	22	20	—	—	—	—	Before
	11	20	3	16	19†	19	One day after
	18	18	15	18	62	17	11 days after
	19	19	3	19	36	22	4 weeks after
85	16	24	4	52	No clots	30'	Before
	27	22	5	37	No clots	21	One week after
	26	25	1	Partial clot in 24 hrs.	Partial clot in 24 hrs.	22	2 weeks after
86	14	31	6	25	60	30	During
	20	25	2	54	No clots	26	One week after
	31	26	None	180	No clots	25	2 weeks after

* Methyl-bis (β -chloroethyl) amine hydrochloride, Merck & Co., 0.1 mgm. per kilogram body weight on four successive days.

† Except for this experiment the improved technique of re-coating the silicone-treated tubes before each use was employed.

Note: All determinations were performed at 37° C.

that the plasma factor can initiate clotting in the absence of platelets.

Recent discovery of several patients with a curious type of circulating anticoagulant (25, 30) lends strong support to the theory of the existence of a plasma thromboplastin precursor. Although the plasmas of these patients contained a potent clotting inhibitor, they reacted in a perfectly normal way to highly diluted thromboplastin. It is apparent, therefore, that the action of the circulating anticoagulant in these cases preceded and in some way prevented the liberation of active thromboplastin. These observations can be explained only by assuming that the clotting inhibitor delays conversion of a thromboplastin precursor to an active thromboplastin.

While our studies show that platelets do not appear to be necessary for the initiation of clotting, it is clear that they increase the rate of clotting and the amount of prothrombin consumed in the process. There is a close correlation between the number of platelets present and the amount of prothrombin converted during clotting in glass tubes. Other observers (5, 6) have previously noted that in thrombocytopenic blood little prothrombin is consumed during clotting. The prothrombin consumed in the coagulation of platelet-free plasma is minimal.

Hemophilic platelet-free plasmas were invari-

ably spontaneously incoagulable in glass tubes at 37° C although they clotted promptly on the addition of thromboplastin. This suggests that the defect in hemophilia is a deficiency of the plasma thromboplastic factor. It is apparent that hemophilia is not caused by any defect in platelets, but rather that the presence of platelets is what makes hemophilic blood clot at all. Even when an untreated hemophilic patient (*e.g.* Case No. 42) has a normal whole blood clotting time, the hemorrhagic diathesis may persist. This indicates that the plasma thromboplastin is necessary for normal hemostasis regardless of the clotting time of the blood *in vitro*. Presumably the "antihemophilic globulin" (31) is identical with the plasma thromboplastin precursor.

Whether or not the plasma thromboplastic factor is totally absent from hemophilic blood has not been established. Lenggenhager (32) believed that it was present in hemophilia but abnormally resistant to activation. Craddock and Lawrence (33) have presented data to indicate that "antihemophilic globulin" may be antigenic in hemophilic subjects. If their observations are correct, it must be presumed that hemophiliacs are entirely devoid of this plasma component.

Quick (34) observed that high-speed centrifugation of oxalated hemophilic plasma caused marked prolongation of the recalcified clotting time.

Quick originally believed that platelets were abnormally resistant in hemophilia, and that they could therefore be removed by centrifugation before thromboplastin was liberated. Rather, it seems to us that the results of Quick's experiment again demonstrate that platelets are critically necessary for the clotting of hemophilic plasma.

Hemophilic blood, presumably lacking in plasma thromboplastic factor, will clot if platelets are present, although clotting is delayed and incomplete. Likewise, normal plasma will clot in the absence of platelets, but in this instance also coagulation is incomplete. It appears that both the platelet and plasma factors are necessary for normal coagulation, although either one alone will suffice to initiate coagulation. The nature of the interaction between platelets and the plasma factor remains to be elucidated.

No evidence was obtained to support the theory that the primary defect in hemophilia is an increased antithromboplastic activity of the plasma. We were unable to demonstrate any antithromboplastic activity of the plasma of our hemophilic patients even when a potent clotting inhibitor was present (Case No. 43).

Normal platelet-free plasma containing an exceedingly low concentration of heparin is spontaneously incoagulable at 37° C even though the prothrombin time upon the addition of thromboplastin is normal. The inverse relationship between heparin activity and platelet concentration has already been reported (24). Studies on the precise mode of action of heparin in platelet-free plasma will be published later.

Experiments on the plasmas of patients with hypoprothrombinemia due to dicumarol gave variable results. In most cases the "platelet-free" plasmas were spontaneously incoagulable in glass tubes. We are unable to account for this on the basis of hypoprothrombinemia alone. Two patients temporarily showed suggestive evidence of circulating anticoagulant. The oxalated plasmas of dicumarolized patients clotted more slowly on the addition of thrombin solution than did normal plasmas. Further studies will be necessary to explain these results.

The "platelet-free" plasmas of most of the patients with thrombocytopenia behaved in a normal manner. However, in each of three cases with prolonged whole blood clotting times the "platelet-

free" plasma was spontaneously incoagulable. We are unable to explain the prolonged whole blood clotting time and associated failure of platelet-free plasma to clot in three cases. It is apparent that these deviations from normal were caused by a deficiency of a thromboplastic factor rather than by the presence of a clotting inhibitor. The numerical reduction of platelets alone is not sufficient to account for the coagulation defect, and we can only surmise that there must have been a deficiency also of a plasma thromboplastin. These plasmas could not be distinguished from those of the hemophiliacs. Other cases of thrombocytopenia with unaccountably prolonged whole blood clotting times have been reported (35).

Two of the three patients with fatal liver disease (Cases Nos. 81 and 82) had positive anti-coagulant assays and their "platelet-free" plasmas did not clot. We have presented evidence that these patients had plasma antithrombic anticoagulants in addition to severe hypoprothrombinemia. By contrast, the "platelet-free" plasma of the third patient (Case No. 83) clotted in glass tubes in spite of a severe hypoprothrombinemia, and this patient had no evidence of circulating anticoagulant.

Three patients with Hodgkin's disease treated with nitrogen mustard showed no evidence of a disturbance of blood coagulation, and their "platelet-free" plasmas were similar to those of normal individuals. The heparin-like anticoagulant reported by Smith *et al.* (26) to occur following nitrogen mustard therapy was not demonstrable in our cases.

SUMMARY

1. By means of silicone-treated apparatus and high-speed centrifugation at low temperature it has been possible to prepare fluid platelet-free plasma without the use of anticoagulants.

2. Studies on the "platelet-free" plasmas of 86 human subjects with and without hemorrhagic diathesis are presented.

3. Evidence is submitted for the existence of a soluble plasma thromboplastin precursor which on contact with glass surfaces is converted to an active state. Both this plasma factor and platelets are necessary for normal coagulation, but clotting can be initiated by either alone.

4. The coagulation defect in hemophilia appears to be a deficiency of this plasma thromboplastin precursor.

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THE HEMODYNAMIC EFFECTS OF HYPOTENSIVE DRUGS IN MAN.¹ I. VERATRUM VIRIDE

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INTRODUCTION

Renewed interest in veratrum viride began with the investigations of Craig and Jacobs (1) who isolated the veratrum alkaloids and found them to be sterol compounds related chemically to the digitalis glycosides, the steroid hormones and vitamin D. Using the pure alkaloids, Kraye and his co-workers (2) demonstrated that sub-toxic doses in experimental animals produced a vasodepressor response of reflex nature. They found that the most important receptor area for the reflex was located in the left ventricle of the heart, and that the primary afferent pathway was the vagus nerve. They did not determine the efferent pathways of this vasodepressor response.

Meanwhile, clinical interest in veratrum viride steadily increased because of its therapeutic use in the eclamptic toxemias of pregnancy (3, 4) and, more recently, in cases of severe essential hypertension (5). Nevertheless, very little was known of its hemodynamic or other pharmacologic effects in human beings. The present study was undertaken to elucidate the actions of the drug in both hypertensive and normotensive individuals.

MATERIALS AND METHODS

The subjects were patients, mostly hypertensive, admitted to the Massachusetts Memorial Hospitals. Both oral (Vertavis³) and parenteral (Veratrone⁴) preparations of veratrum viride were administered. Arterial pressure was measured either with a Hamilton manometer (6) (during the determinations of vasopressor responses, cardiac output, and/or hepatic-portal blood

flow); or with an arm cuff and a mercury manometer. Cardiac output was determined directly by the Fick principle using the intravenous catheter method of Courmand (7) and determinations of blood oxygen by the method of Van Slyke and Neill (8). In order to insure complete mixing, the venous blood samples were withdrawn always from the pulmonary artery, while oxygenated blood was taken either from the brachial or the femoral artery. Ballistocardiograms (9, 10) were recorded simultaneously in these and other hypertensive subjects but due to the abnormal ballistocardiographic complexes occurring in patients with hypertension, accurate measurements of cardiac output by this method were not possible. Blood flow in the forearm and calf was determined plethysmographically by the method of Wilkins and Eichna (11), modified by the use of a thin rubber sleeve instead of the rubber diaphragms cemented to the skin (12). Hepatic-portal (splanchnic exclusive of renal and adrenal) blood flow was estimated by the bromsulphalein method of Bradley *et al.* (13), with three modifications: first, peripheral blood samples consistently were arterial; second, determinations of BSP were made on oxalated plasma rather than serum; and third, estimations of blood flow rate were calculated for the times at which arterial blood samples were drawn. Renal plasma flow (PAH) and glomerular filtration rate (mannitol, inulin) were determined by the methods of Smith and his co-workers (14).

Vasopressor overshoots after blood-pressure-lowering procedures were measured by the method of Wilkins and Culbertson (15), reflex vasoconstrictions in the digits by the method of Bolton, Carmichael and Stürup (16) and skin temperature responses according to the method of Uprus, Gaylor and Carmichael (17). Vascular distensibility in the extremities was determined using the method of Litter and Wilkins (18).

RESULTS

I. Cardiac function

Cardiac output, mean arterial pressure and total peripheral resistance

Control and experimental determinations were carried out during a single test on five patients given Veratrone by intramuscular injection. In two other patients a control study was done, after

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³ Irwin, Neisler & Company, Decatur, Illinois.

⁴ Parke, Davis & Company, Detroit, Michigan.

TABLE I
Effects of veratrum viride on blood pressure, cardiac output and total peripheral resistance

Patient and diagnosis	Sex	Age	Drug and dose	Control					After veratrum					
				Mean arterial pressure	Cardiac rate	Cardiac output	Total peripheral resistance	Pulmonary arterial pressure	Time from administration of drug	Mean arterial pressure	Cardiac rate	Cardiac output	Total peripheral resistance	Pulmonary arterial pressure
W. G. Essential hypertension	M	52	Vertavis* 40 Craw units	mm. Hg† 166.5 167.5	per min. 92 97	L. per min. 6.66 6.44	dynes cm.-5 sec. 2010 2040	mm. Hg 32/15 30/18	minutes 232 244	mm. Hg 119 130	per min. 86 88	L. per min. 7.30 6.70	dynes cm.-5 sec. 1302 1483	mm. Hg 21/16 21/14
E. S. Essential hypertension	F	46	Vertavis 40 Craw units	185.5 183	112 108	6.21 5.36	2394 2730	26/8 23/7	110 120	135 141	94 93	6.94 5.60	1557 2012	18/8 19/8
S. B. Essential hypertension	M	48	Veratrone† 1.0 cc.	162.5	84.5	5.83	2227	21/7	34 94	146 158.5	77 79	6.10 6.24	1912 2031	18/5 21/8
J. Mc. Essential hypertension with cardiac failure	M	52	Veratrone 1.0 cc.	225 218	139 133	4.22 4.28	4210 4075	95/62 87/60	38 74	177 174	108 106	4.95 5.57	2855 2500	55/26 60/30
M. H. Essential hypertension	F	49	Veratrone 0.6 cc.	190	86	5.75	2640	25/8	82	166.5	73	5.01	2660	23/9
W. C. Malignant hypertension with cardiac failure	M	50	Veratrone 0.4 cc.	175 180	82 82	3.98 3.77	3460 3820	65/36 69/36	11 13	154 138	78 63	4.39 4.94	2800 2200	66/34 41/20
J. C. Malignant hypertension with cardiac failure	M	48	Veratrone 0.45 cc.	166.5 165.5	97 94	5.98 5.47	2220 2420	46/22 37/19	17 27	159.5 157	90 85	5.80 5.16	2190 2420	27/10 22/4

* Oral veratrum viride given twice per day for one week after control test in four equal hourly doses (time counted from beginning of last dose).

† Parenteral—given intramuscularly immediately following control period.

‡ Determined by planimetric measurement of the arterial pulse waves.

EFFECT OF VERATRUM ON PULSUS ALTERNANS AND BCG

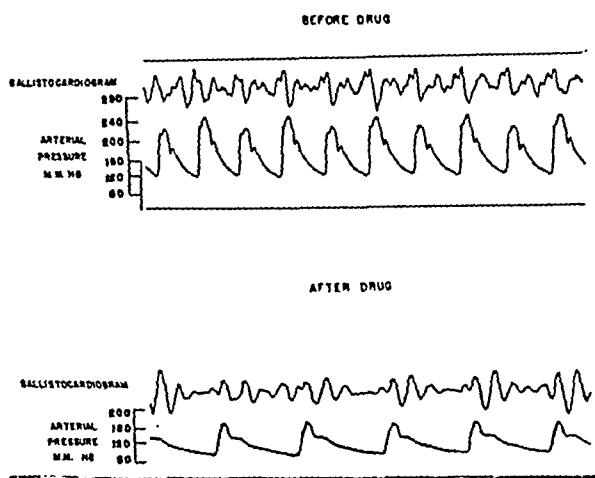


FIG. 1. OPTICAL RECORDS OF THE BALLISTOCARDIOGRAM AND THE BRACHIAL ARTERIAL PRESSURE (HAMILTON) BEFORE AND AFTER THE ORAL ADMINISTRATION OF 30 CRAW UNITS OF VERATRUM VIRIDE IN PATIENT M. A., MALE, AGED 43

Pulsus alternans was present in the control record. Following veratrum there was a marked reduction in arterial pressure, a disappearance of pulsus alternans, and a return of the ballistocardiogram to a more normal pattern.

which Vertavis was administered orally for one week before the determinations were repeated. The results in both types of experiments were essentially the same (Table I). In all but two of the

seven cases there was a fall in mean arterial pressure of from 8 to 28 per cent, and in three patients, of more than 20 per cent. However, during the period of reduced blood pressure depression of cardiac output was not observed. Rather, in three cases, there was a slight increase in cardiac output of 6 to 8 per cent, and in two patients, with congestive heart failure, an increase of 20 and 24 per cent. As a result of the significant fall in mean arterial pressure and slight rise in cardiac output there was a considerable decrease in the calculated total peripheral resistance (between 30 and 35 per cent in four of the cases and of 11 per cent in one case). In two cases (M. H. and J. C., Table I) there were insignificant changes in cardiac output or peripheral resistance during the time the cardiac output determinations were being carried out, the major portion of the test period in patient M. H. having been devoted to estimations of hepatic-portal blood flow (Table III, Figure 5).

Other aspects of cardiac function

It was not unusual to observe a decrease in cardiac enlargement as measured by roentgenography (5) in patients who exhibited a prolonged reduction in blood pressure after months of continuous oral administration of veratrum viride. Less frequently, changes toward normal in the electrocardiogram were observed. Pulsus alternans usually decreased or disappeared during the reduc-

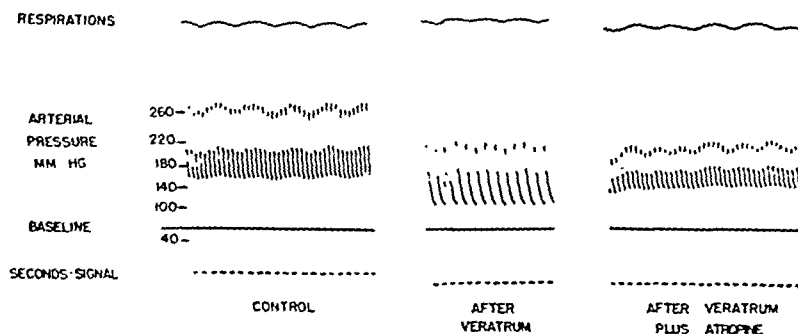


FIG. 2. OPTICAL RECORDS OF RESPIRATION AND BRACHIAL ARTERIAL PRESSURE (HAMILTON) IN PATIENT C. B., FEMALE, AGED 49

Ninety minutes following the oral ingestion of 20 Craw units of veratrum viride (second tracing) there was a fall in arterial pressure accompanied by bradycardia. Atropine (1 mgm. intravenously) was given immediately, and three minutes later (third tracing) there was a tachycardia and a rise in diastolic but not in systolic pressure. The diastolic rise was more apparent than real since it was associated with shortening of the run-off period.

TABLE II
Effect of veratrum viride (Veratrone) on blood flow in forearm and calf*

Case no.	Patient and diagnosis	Sex	Age	Dose	Control			After veratrone			
					Arterial pressure	Blood flow per 100 cc. limb volume		Time after drug	Arterial pressure	Blood flow per 100 cc. limb volume	
						Arm	Leg			Arm	Leg
					mm. Hg	cc.	cc.	minutes	mm. Hg	cc.	cc.
1	D. C. Essential hypertension	F	27	0.6 cc.	180/112 173/115 170/116	2.83 2.56 3.41	3.01 2.77 2.78	11	158/100	2.85	2.19
								12	141/90	2.73	2.02
								34	111/73	2.25	1.69
								52	103/71	2.24	1.77
								77	115/81	2.95	2.40
								97	121/88	2.84	2.37
								107	132/93	2.86	2.69
								134	137/95	3.38	3.01
2	L. B. Essential hypertension	F	49	0.5 cc.	160/100 156/110	3.29 2.74	2.54 2.57	13	134/106	2.68	2.63
								34	132/99	2.39	1.91
								46	129/95	1.97	1.73
								67	—	2.46	2.21
				0.15 cc. at 70 minutes				87	114/93	2.37	2.47
								99	147/94	2.98	2.56
								109	149/100	2.59	2.27
3	M. St. J. Essential hypertension	F	51	0.5 cc.	196/120 198/116	3.92 3.94 4.04	2.54 2.42 2.40	20	190/116	3.40	2.42
				0.2 cc. at 35 minutes				57	164/110	2.22	2.30
								74	160/104	3.28	2.43
								85	158/101	3.11	2.52
4	D. C. Essential hypertension	M	50	0.5 cc.	201/121 175/112	1.49 1.86	— —	50	181/113	2.18	—
				0.2 cc. at 55 minutes				72	179/110	2.76	—
								97	156/107	2.48	—
3	F. V. Chronic glomerulonephritis advanced renal damage	M	22	0.5 cc.	175/114 167/116	2.23 2.46	2.66 2.73	23	166/106	—	2.96
								36	164/98	2.88	2.89
				0.15 cc. at 50 minutes				55	162/99	2.81	2.82
								68	152/92	2.54	2.98
								79	152/88	2.54	2.73
								91	154/85	2.72	3.04
6	R. F. Essential hypertension advanced renal damage	M	46	0.75 cc.	199/130 186/122 189/120	1.70 2.10 2.16	3.29 2.86 2.76	13	193/120	2.49	3.08
								25	170/109	2.50	2.82
								39	136/88	2.16	2.27

* Parenteral—given intramuscularly immediately following control period.

tion of blood pressure following veratrum viride, and the ballistocardiogram also occasionally reverted toward a more normal form (Figure 1).

Pulmonary congestion lessened and dyspnea improved during the hypotensive action of veratrum. For example, patient J. Mc. (Table I) at the time of the control cardiac output determination exhibited clinical evidences of cardiac failure, including dyspnea and pulmonary congestion. After Veratrone, the systemic arterial pressure de-

clined from 290/185 to 230/135 while the pulmonary arterial pressure fell from 90/60 to 58/28 mm. Hg and the cardiac stroke volume increased from 32 to 48 ml. At this time the patient noted subjective relief of dyspnea. The injection of Veratrone was followed by a decrease in pulmonary arterial pressure in the three cases who exhibited abnormal elevations (Table I).

Bradycardia was frequently observed after veratrum viride and was shown to be vagal in origin

by the fact that it could be abolished by the intravenous injection of 1 mgm. of atropine. Although atropine abolished the bradycardia it did not greatly affect the reduction in blood pressure (Figure 2). In occasional patients bradycardia was not manifested following Veratrum even though the arterial blood pressure was significantly reduced (case W. G., Table I). Thus, bradycardia appeared to be a common, perhaps an augmenting, but not a necessary accompaniment of the hypotensive response.

II. Blood flow through various regions

Blood flow through the forearm and calf

All six hypertensive patients who were studied plethysmographically following the intramuscular injection of Veratrone (Table II), had a decrease in mean arterial pressure of from 8 to 38 per cent. The blood flow to the forearm in two of the six subjects decreased as the arterial pressure fell, but returned to control levels as the arterial pressure became stabilized at a lower level. In three subjects, as the pressure fell the blood flow in the forearm increased and remained elevated, while in one the flow was unchanged. The blood flow in the calf followed a similar pattern, falling in two of five cases but returning to control levels before the blood pressure, increasing in two, and remaining unchanged in one. Since in all of these patients

blood flow remained at or returned to or above control values during the hypotensive response to Veratrone a decrease in peripheral resistance in the limbs was assumed to have occurred.

Blood flow through the hepatic-portal circuit

The blood flow through the liver (EHBF) was estimated in three hypertensive patients in the basal state and horizontal position both before and after administration of veratrum viride (Table III). In the first patient, W. G., two separate studies were made, one for control measurements and the other after a week of treatment with Vertavis tablets by mouth. The latter study revealed an increase of 25 per cent in the average EHBF and a decrease of 27 per cent in the average "mean" ($= \frac{S + D}{2}$) arterial pressure, indicating a significant reduction in hepatic-portal vascular resistance.

In the two other cases acute experiments were performed. After a period of control observations, patient J. C. received 0.6 ml. of Veratrone solution intramuscularly. Twenty minutes later, because there had been no evidence of a hypotensive response, but on the contrary, a moderate pressor reaction, he received a dose of 0.2 ml. of Veratrone. Twelve minutes after the second injection, EHBF and arterial pressure had decreased from control values by 12 per cent and 32 per cent respectively, and at 22 minutes by 21 and 46 per cent respec-

TABLE III
Effects of veratrum viride on estimated hepatic blood flow

Patient and diagnosis	Sex	Age	Drug and dose	Control		After veratrum		
				Arterial pressure	EHBF	Elapsed time	Arterial pressure	EHBF
				mm. Hg	ml. per min. per 1.73 sq. m.	minutes	mm. Hg	ml. per min. per 1.73 sq. m.
W. G. Essential hypertension	M	52	Vertavis* 40 Craw units	235/142	1106	282	183/95	1590
				230/140	1307	293	177/90	1411
						306	145/90†	1521
J. C. Essential hypertension	M	50	Veratrone† 0.8 cc.	219/121	1104	15	230/130	1126
				215/120	1269	24	240/130	1285
				213/120	1183	32	150/80	1040
						42	120/60	931
M. H. Essential hypertension	F	49	Veratrone 0.7 cc.	220/133	925	15	210/120	1130
				224/141	1154	31	183/105	960
				220/135	1243	42	177/106	1129
						54	184/110	1050
						60	185/112	1260

* † Notations as in Table I.

† Clot in needle prevented accurate recording of arterial pressure.

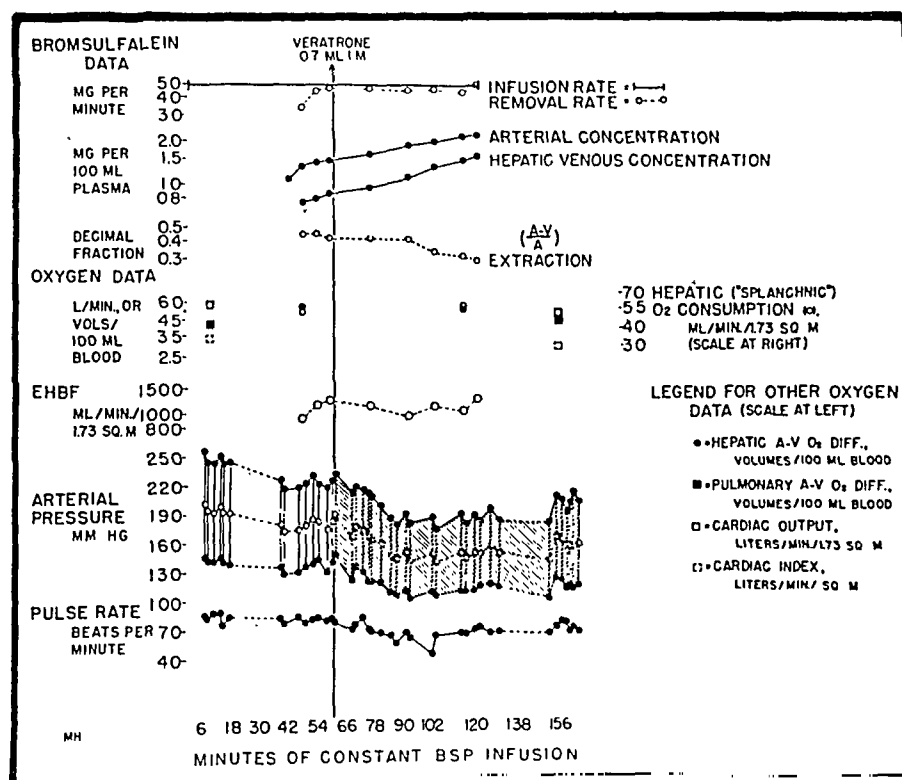


FIG. 3. CHART OF ESTIMATED HEPATIC-PORTAL BLOOD FLOW (EHBF), ARTERIAL PRESSURE (HAMILTON), AND PULSE RATE IN PATIENT M. H., FEMALE, AGE 50

Above are the infusion and removal rates, the arterial and hepatic venous concentrations and the extraction rate of bromsulfalein. In the middle are data on hepatic and pulmonary A-V oxygen differences, cardiac output (Fick), cardiac index and hepatic oxygen consumption. Observed data are represented by solid symbols and lines, calculated values by open symbols and lines. Following the injection of 0.7 ml. of Veratrone (vertical line) there was a significant fall in arterial pressure. The estimated hepatic blood flow fell off slightly during the first 30 minutes after the drug and then increased above the highest level recorded in the control period.

tively. Thus, a reduction in hepatic-portal vascular resistance became apparent, but the hypotensive reaction then became excessive, and proceeded to a state of collapse with arterial pressure of 90/48 mm. Hg, pulse rate of 75 beats per minute, and development of nausea and vomiting. Therefore, experimental observations were discontinued and restorative therapy was instituted. While this case illustrates acute hemodynamic changes strikingly, it serves also as an example of the effects of overdosage.

The third patient, M. H., was observed similarly before and after a single injection of 0.7 ml. of Veratrone, which in this instance proved to be less than the optimal therapeutic dose and produced only a modest reduction in arterial pressure (Table III, Figure 3). Nevertheless, EHBF re-

mained above the lowest control value and finally rose above the highest control value at a time when "mean" arterial pressure had become stabilized at a level definitely (17 per cent) below the average control. Hence, this case also is consistent with the others in indicating that a significant reduction occurs in hepatic-portal vascular resistance following treatment with veratrum viride.

Blood flow through the kidneys

Changes in renal blood flow were similar to those observed in hepatic blood flow after veratrum viride. In two (W. G. and A. D.) of three cases maintained for one week on continuous oral administration of the drug (Table IV), the effective renal plasma flow increased moderately despite significant reductions in arterial pressure. In the

TABLE IV
Effects of veratrum viride on renal clearances

Patient and diagnosis	Sex	Age	Drug and dose	Control					After veratrum					
				Arterial pressure	Plasma clearance mannitol cc. per min. per 1.73 sq. m.	Plasma clearance PAH cc. per min. per 1.73 sq. m.	Filtration fraction per cent	Urine flow cc. per min.	Time after drug administration minutes	Arterial pressure mm. Hg	Plasma clearance mannitol cc. per min. per 1.73 sq. m.	Plasma clearance PAH cc. per min. per 1.73 sq. m.	Filtration fraction per cent	Urine flow cc. per min.
W. G. Essential hypertension	M	52	Vertavis* 40 Craw units	219/126	142	330	38.5	2.2	238	154/90	73	410	17.8	1.9
				215/132	104	367	33.1	2.4	282	183/95	71	369	19.2	1.6
				235/142		314			293	177/90	62	313	19.8	1.6
				230/140		306			306	145/90†	72	414	17.4	1.6
D. W. Essential hypertension	F	38	Veratrone† 0.6 cc.	200/108	110	425	25.9	1.3	17	98/55	37	238	15.5	0.7
				208/105	104	422	24.7	1.2	29	94/60	33	256	12.9	0.5
				204/104	112	492	23.0	1.1	44	115/60	104	587	17.7	2.1
G. A. Essential hypertension	F	42	Veratrone 0.7 cc.	214/126	132	487	27.2	9.4	28	170/110	90	414	21.8	2.2
									48	165/105	87	382	22.8	1.7
				218/128	122	441	27.6	7.4	72	160/105	66	306	21.6	1.3
S. B. Essential hypertension	M	48	Veratrone 1.0 cc.						89	165/108	100	443	22.5	1.8
									10	228/118	124	370	33.5	7.2
				228/116	134	424	31.6	11.0	31	222/118	93	411	22.7	4.8
				222/114		412		10.9	51	228/116	66	358	18.4	4.1
									73	200/102	89	386	23.0	2.9
									92	216/110	73	373	19.6	2.3

TABLE IV—Continued

Patient and diagnosis	Sex	Age	Drug and dose	Control					After veratrum					
				Arterial pressure	Plasma clearance inulin <i>cc. per min. per 1.73 sq. m.</i>	Plasma clearance PAH <i>cc. per min. per 1.73 sq. m.</i>	Filtration fraction <i>per cent</i>	Urine flow <i>cc. per min.</i>	Time after drug administration <i>minutes</i>	Arterial pressure <i>mm. Hg</i>	Plasma clearance inulin <i>cc. per min. per 1.73 sq. m.</i>	Plasma clearance PAH <i>cc. per min. per 1.73 sq. m.</i>	Filtration fraction <i>per cent</i>	Urine flow <i>cc. per min.</i>
G. J. Chronic glomerulo-nephritis	M	47	Veratrone 0.7 cc.	<i>mm. Hg</i> 192/120 188/118	65 57	285 296	20.1 17.1	8.3 7.4	13 25 46 66 90 116	170/112 178/110 150/90 148/88 150/90 162/94	40 67 64 62 75 97	141 202 204 243 283 366	24.6 29.2 27.3 22.2 23.0 23.7	2.7 1.4 0.8 0.6 0.7 0.8
				190/130 187/132 190/134	44 38 28	204 135 128	19.7 25.8 20.2	2.8 2.3 1.3	6 16 38 57 86	180/125 164/102 160/90 155/100 146/95	23 28 25 29 48	122 119 117 155 228	17.6 21.4 19.7 17.3 19.3	1.0 0.7 0.6 0.7 0.8
				190/95 194/90 200/95 200/95	109 124 130	380 454 496 542	24.0 25.0 24.0	5.1 5.0 3.1 2.5	8 18 28 43 51 66	195/92 170/78 160/72 140/68 136/68 140/72	86 63 65 63 109 105	375 266 350 294 640 630	22.9 23.7 18.6 21.4 17.0 16.7	1.4 0.3 0.3 0.2 0.4 0.4
M. G. Essential hypertension	M	49	Veratrone 0.6 cc.	212/116 190/116 190/116	116 108 112	568 416 485	20.5 25.6 23.0	9.4 4.0 2.5	10 30 60	205/125 140/95 164/100	66 87 105	275 375 590	24.8 23.0 17.8	1.6 0.3 0.7
				230/114 226/116 226/116	43 50 37	147 141 126	34.2 38.2 34.1	8.4 7.1 7.2	70* 82 92	190/86 190/88 180/86	23 29 28	143 133 138	16.1 21.9 19.2	2.6 2.5 2.4
A. D. Essential hypertension	F	68	Vertavis* 30 Craw units	188/104 186/102	53 47	342 238	18.0 21.4	4.3 2.5	110* 121 131	116/68 124/74 130/76	57 74 50	322 416 263	17.8 17.7 18.9	3.3 4.0 2.6

* † Notations as in Table I.

‡ Notation as in Table III.

third (B. H.) it remained unchanged. In all of seven cases who received veratrum viride acutely by intramuscular injection (Table IV) there was an initial decrease in renal plasma flow followed in the later period by a return to control values. In five of these cases renal blood flow after veratrum rose to levels above those recorded in the control period (Figure 4, Table IV).

Estimations of cardiac output, hepatic-portal and renal blood flows were carried out in a single subject (case W. G., Tables I, III and IV) both before and after one week of treatment with orally administered veratrum viride. Following the cardiac output determinations the intravenous catheter was moved from the right pulmonary artery to an hepatic vein, urine samples being obtained meanwhile through a bladder catheter. These procedures permitted estimation of the percentage of the cardiac output circulating through the hepatic-portal and renal vascular beds before and after veratrum viride. In the control period the mean cardiac output was 6.55 L. per min., the average estimated hepatic-portal blood flow was 1.2, and the mean renal blood flow was 0.565 L. per min. After one week of continuous oral administration

of veratrum viride the mean values were for cardiac output 7.0 L. per min., for estimated hepatic-portal blood flow 1.48 L. per min., and for renal blood flow 0.612 L. per min. Thus, estimated hepatic-portal blood flow utilized 18.3 per cent of the cardiac output before the drug and 21.7 per cent after the drug; renal blood flow utilized 8.6 per cent of cardiac output prior to treatment and 8.7 per cent after treatment. These results suggest that despite a reduction in arterial pressure from approximately 225/130 to 170/92 the percentage of the cardiac output utilized by the hepatic-portal and renal vascular beds showed no significant change.

III. Renal function

In addition to the studies of renal plasma flow just described, measurements of glomerular filtration, filtration fraction and urine volume were also made (Table IV). Four of the patients exhibited advanced renal disease characterized by abnormally low clearances of inulin and para-aminohippurate.

Mannitol clearance following veratrum appeared to fall and remain low. Inulin clearance

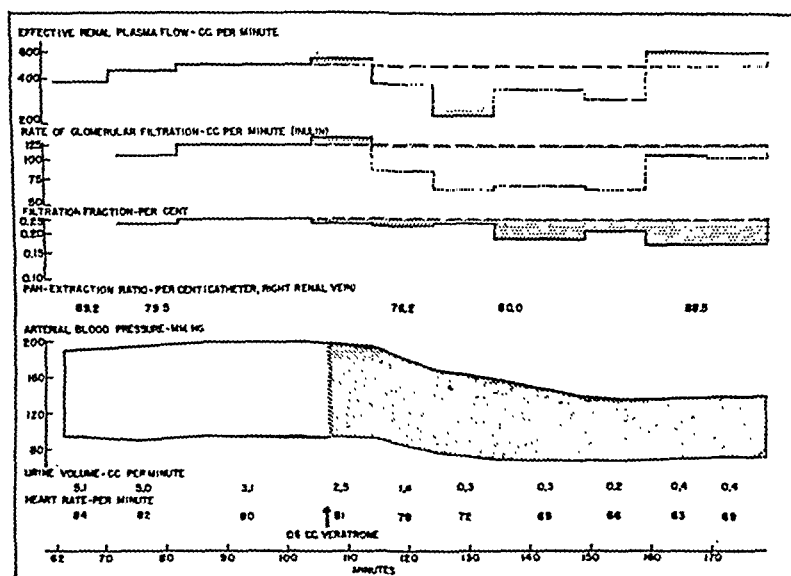


FIG. 4. CHART OF RENAL PLASMA FLOW, GLOMERULAR FILTRATION RATE, FILTRATION FRACTION, RENAL EXTRACTION OF PAH, BRACHIAL ARTERIAL PRESSURE, URINE VOLUME AND HEART RATE BEFORE AND AFTER INTRAMUSCULAR VERATRUM IN PATIENT E. S., FEMALE, AGED 44

Oliguria appeared early, and remained marked even when the renal clearances had returned to control levels. Note relatively constant extraction of PAH.

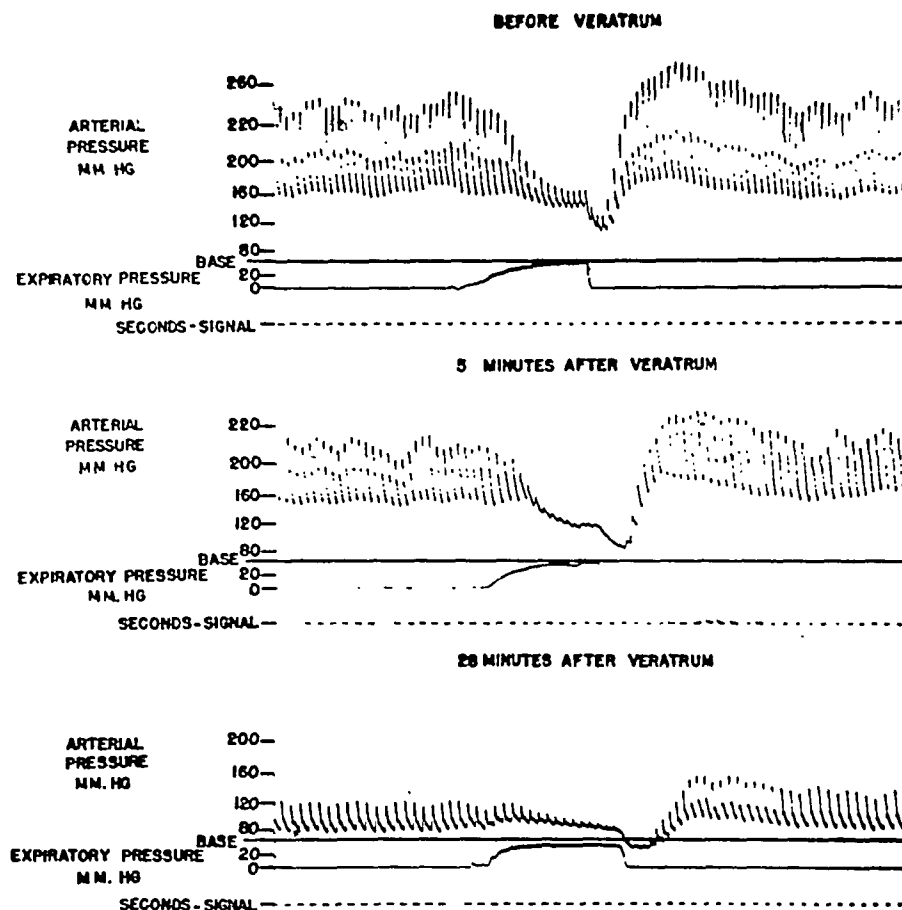


FIG. 5. OPTICAL RECORDS OF THE RESPONSE IN ARTERIAL PRESSURE (HAMILTON) TO THE VALSALVA MANEUVER IN PATIENT C. B., FEMALE, AGED 49, BEFORE AND AFTER VERATRUM VIRIDE

Despite a marked reduction of the basal arterial pressure the overshoot response after the Valsalva was not blocked by veratrum.

first fell and then returned approximately to control values in five patients (Table IV) while in one patient (B. H., Table IV), who had marked impairment of renal function and low clearances in the control period, the inulin clearance fell and remained below the control values.

In contrast to the relatively moderate fluctuations in effective renal plasma flow, in glomerular filtration rate (inulin) and in filtration fraction, the urine volume was invariably and often strikingly reduced after the parenteral injection of veratrum viride (Table IV, Figure 4). The oliguria was associated with a marked increase in the U/P ratio of inulin or mannitol, clearly indicating that the diminished urine flow was due to increased tubular reabsorption of water. The marked rise in U/P ratio of para-aminohippurate was shown in one case (E. S., Table IV, Figure 4) to be due to increased water reabsorption exclusively since in this case the renal extraction of PAH as measured directly in the renal venous blood did not change

despite marked oliguria and more than tenfold increase in urine concentration of PAH.

Oliguria occurred after parenteral injections of Veratrone even when there was no perceptible reduction in blood pressure. This was demon-

TABLE V
Vasopressor responses to the valsalva maneuver before and after the administration of veratrum viride

Patient	Control			After veratrum		
	Arterial pressure		Over-shoot*	Arterial pressure		Over-shoot
	Basal	After valsalva		Basal	After valsalva	
I. B.	mm. Hg 282/182	mm. Hg 308/188	per cent 7	mm. Hg 228/156	mm. Hg 256/180	per cent 14
M. S.	240/130	290/178	26	194/102	263/132	33
M. A.	222/123	261/142	17	110/60	124/62	9
J. Mc.	270/160	290/180	9	170/105	178/111	6
J. F.	257/110	290/110	9	180/88	168/81	0
C. B.	238/158	272/170	11	120/76	160/108	37

* Calculated from the "mean" (one-half the sum of systolic and diastolic) pressure.

TABLE VI

The vasopressor responses to the quick tiltback from the upright to the horizontal position before and after the administration of veratrum viride

Patient	Control				After veratrum			
	Arterial pressure			Overshoot*	Arterial pressure			Overshoot
	Basal	After standing 5 min.	Tiltback overshoot		Basal	After standing 5 min.	Tiltback overshoot	
	mm. Hg	mm. Hg	mm. Hg	per cent	mm. Hg	mm. Hg	mm. Hg	per cent
C. B.	273/170	274/180	295/182	8	118/80	98/76	130/85	9
M. A.	221/132	193/129	260/148	16	112/69	99/76	155/80	30
J. Mc.	277/172	277/187	268/174	0	158/88	120/94	154/90	0
J. F.	260/112	262/117	280/115	6	194/89	176/94	202/90	3

* Calculated from the "mean" (one-half the sum of systolic and diastolic) pressure.

strated in two normotensive subjects in whom 5 per cent glucose in water was administered intravenously at a constant rate until a steady state of urine excretion was obtained as determined by washing out the bladder at ten-minute intervals. A dose of 0.4 cc. of Veratrone caused no significant change in blood pressure, but did reduce the urine volume from 5.45 and 8.33 cc. per minute to 1.02 and 4.53 cc. per minute, respectively, in the two patients. Oliguria was not observed when the reduction in blood pressure was maintained by the continued oral administration of veratrum viride (patients W. G. and A. D., Table IV).

IV. Other hemodynamic functions

Cardiovascular reflexes—Sympathetic responses

Unlike sympatholytic agents, veratrum viride did not abolish reflex sympathetic vasoconstriction. In six hypertensive subjects the vasopressor overshoot following the Valsalva maneuver was increased in three cases, slightly decreased in two, and abolished in one after as compared with before veratrum viride (Table V, Figure 5). Postural hypotension was not observed in four subjects who were tilted to an angle of 75° for five minutes (Table VI). The hypertensive overshoot on the tilt back from the erect to the supine position was increased in two of these cases, decreased in one and not present in one case (Table VI). Postural hypotension occurred in only two of 54 patients treated clinically with veratrum viride by mouth (5). Postural hypotension with collapse never occurred with therapeutic doses of the drug.

In contrast with the depression of the cold pres-

sor response after certain sympatholytic agents (19-21) there was an augmentation after veratrum viride in two of four subjects examined and no change in the other two (Table VII). Similarly, the hypertensive response evoked in a subject by the emotional stimulus of a problem in mental arithmetic was not significantly altered during veratrum hypotension.

Reflex vasoconstriction in the digits was examined in two patients before and after the injection of veratrum viride. Finger plethysmographic recordings of the vasoconstrictor responses to a deep breath, the application of ice to the forehead and pin prick on the skin were if anything increased after veratrum viride. Further, in four patients examined in a room maintained at a constant temperature of 68° F there was no change in the skin temperature of the digits after the administration of Veratrone despite the development of marked

TABLE VII

Responses to the cold pressor test before and after the administration of veratrum viride

Patient	Control			After veratrum		
	Arterial pressure		Increase*	Arterial pressure		Increase
	Basal	Peak response to ice		Basal	Response to ice	
	mm. Hg	mm. Hg	per cent	mm. Hg	mm. Hg	per cent
J. F.	287/113	297/118	4	191/88	238/108	24
J. Mc.	282/168	325/194	15	167/100	228/136	36
M. S.	230/145	270/180	20	145/95	180/110	21
P. P.	238/145	300/200	30	143/95	210/134	44

* Calculated from the "mean" (one-half the sum of systolic and diastolic) pressure.

TABLE VIII
Effect of veratrone on blood flow in sympathectomized extremities

Case no.	Patient and diagnosis	Sex	Age	Drug and dose	Control			After veratrone			
					Arterial pressure	Blood flow per 100 cc. limb volume		Time after drug	Arterial pressure	Blood flow per 100 cc. limb volume	
						Lt. arm	Lt. leg*			Lt. arm	Lt. leg*
1	M. Q. Essential hypertension lumbodorsal splanchnicectomy through L2 lower extremities sympathectomized	F	35	Veratrone 0.5 cc.	m. Hg 209/136	cc. 1.45	cc. 1.76	6	mm. Hg 205/149	cc. 1.78	—
								18	185/125	1.96	—
								22	158/114	2.31	—
								31	142/106	2.10	—
								43	122/92	1.79	1.11
								67	144/110	1.63	1.13
								89	171/117	1.96	1.13
								101	170/125	1.90	1.13
2	L. O'S. Essential hypertension right transthoracic sympathectomy	F	24	Veratrone 0.5 cc.		Rt.* arm	Lt. arm			Rt.* arm	Lt. arm
								3	253/159	2.03	1.65
						1.70	1.43	24	213/134	1.50	1.78
						1.68	1.49	41	181/123	1.37	1.82
						1.64	1.58	59	175/123	1.33	1.78
3	E. R. Essential hypertension right lumbar sympathectomy right lower extremity sympathectomized	F	40	Veratrone 0.7 cc.		Rt.* leg	Lt. leg			Rt.* leg	Lt. leg
								8	179/105	1.77	1.42
						1.68	1.16	23	146/93	1.80	1.42
						1.78	1.07	38	129/81	1.90	1.50
								43	129/83	1.71	1.35
								65	134/84	1.69	1.32
								86	146/89	1.88	1.20

* Sympathectomized.

reductions in blood pressure. These various observations show that the drug did not block sympathetic reflexes.

However, evidence that the sympathetic nervous system may be involved in some way in the depressor response was suggested by the data regarding blood flow through sympathectomized extremities. The effect of Veratrone on the blood flow in sympathectomized as contrasted with normally innervated extremities was studied in five patients. Three, who were hypertensives, manifested a significant hypotensive response to the drug (Table VIII). Two of them showed a persistent decrease in blood flow in the sympathectomized extremity, whereas the flow in the normally innervated control limb was increased. In the third patient there was a slight increase in blood flow in both extremities, greater in the normally innervated limb, but the results may have been affected by the presence of considerable pain. In the two normotensive subjects similarly studied

after veratrum viride there were insignificant changes in both blood flow and arterial pressure.

Reaction to ephedrine and epinephrine

Veratrum did not abolish the pressor response and rise in heart rate following the injection of either epinephrine or ephedrine. The intramuscular injection of 0.05 gm. ephedrine promptly abolished the collapse reaction observed in three patients who were given an overdose of veratrum viride.

Vascular distensibility in the extremities

The vascular distension that occurs during venous congestion of the extremities (18) was measured in the forearm and calf in eight hypertensive and three normotensive patients before and after the intramuscular injection of Veratrone. The distension of the forearm segment in the plethysmograph during inflation of a congesting cuff to 30 mm. Hg was definitely greater after

Veratrone than before in five of 11 patients. In four the vascular distensibility was only slightly greater and in two there was no change. In the calf of one of six subjects studied there was a marked increase in vascular distensibility, in three a slight increase, and in two no change. No qualitative differences were observed between the normotensive and hypertensive cases.

Vascular distensibility measured in the sympathectomized lower extremities of two patients did not increase after Veratrone whereas in the normally innervated upper extremities it showed a marked increase. In the sympathectomized forearm of one patient there was a minimal increase in vascular distensibility after Veratrone while in the contralateral normally innervated forearm there was a marked increase.

These changes in the vascular distensibility after Veratrone occurred early and persisted for the duration of the experiments. They were usually greater and occurred earlier in the forearm than in the calf. There was little correlation between these changes and the alterations in blood pressure, blood flow, or calculated peripheral resistance.

Systemic response to venous congestion of the extremities

Previous observations in this laboratory have demonstrated that sodium nitrite and various sympatholytic agents alter markedly the systemic response of normal and hypertensive subjects to venous congestion of the extremities (22). In this test, the legs and one arm of the supine subjects were congested by cuffs placed proximally and inflated to pressures of 100 mm. Hg. After a hypotensive dose of sodium nitrite or of various sympatholytic drugs, patients frequently developed marked hypotension and collapse within two to five minutes following the application of the congesting pressure. By contrast, despite marked reductions in the resting blood pressure after veratrum, the application of such congesting pressures to the extremities for a period of five minutes was well tolerated in five subjects.

DISCUSSION

The hypotensive response to veratrum viride occurred in two phases: (1) an initial adjustment phase followed by (2) a more stable hypotensive

phase. The first phase was characterized by sharp, and often fluctuating, decreases in arterial pressure, pulse rate and peripheral blood flow. The second phase consisted of a steady state of reduced arterial pressure and pulse rate, with a return of hepatic, renal and muscle blood flows to control values. Cardiac output in the second phase was found to be essentially unchanged from the pretreatment control values. These findings along with the decreased arterial pressure were accepted as indicating a decrease in peripheral resistance.

The decrease in peripheral resistance after veratrum viride appeared to occur in all the areas under study including the retinal arterioles (5, 23). Compensatory tachycardia and palpitation of the type produced by the peripherally-acting vasodilators such as sodium nitrite or tetraethylammonium salts did not appear, nor did postural collapse occur with sub-toxic doses of the drug. Vasopressor responses to the Valsalva experiment, the erect posture, the cold pressor test and emotional stimuli remained intact. Thus, a state of circulatory equilibrium was achieved with normal cardiac output and blood flow to the vital organs in spite of the lower arterial pressure. This integrated type of hypotensive response after the crude drug was similar to that observed in experimental animals after the administration of the pure alkaloids of veratrum (2), suggesting that it too was mediated through nervous reflexes involving the higher vasomotor centers.

While the second, stable phase of the hypotensive reaction to veratrum viride was characterized by generalized vasodilatation and a return of blood flow to normal, the initial falling phase of the reaction appeared to involve different mechanisms since it not infrequently was associated with parallel decreases in peripheral blood flow. Therefore, the initial fall in arterial pressure could not always be due to a generalized decrease in peripheral resistance. A depression of cardiac output might well account for the initial fall in arterial pressure. It need not be primary in the heart but could result from a temporary failure of venous return due to dilatation of the post-arteriolar vascular beds and a consequent relative shift of circulating blood volume from the arteries to the capillaries and veins. Neither need a depression in cardiac output be sizable to cause a marked fall in arterial pressure provided it continue for some minutes. It is

known that large decreases in cardiac output such as occur with the Valsalva experiment are capable of lowering the arterial pressure of hypertensive patients to normal within a few seconds (15). An immeasurable decrease in cardiac output therefore might cause a similar effect if continued over a longer period of time such as that required for the arterial pressure to stabilize at a lower level after veratrum. Studies now in progress (24) on the changes in cardiac output during the initial phase of the hypotensive action of veratrum may reveal decreases of cardiac output to occur, but in the light of the above argument, they may not.

The oliguria which followed the parenteral injection of veratrum viride appeared to be independent of changes in either glomerular filtration rate or renal plasma flow, since suppression of urine volume continued to be most marked when these measurements had returned to or above control values. The oliguria seemed to be independent also of the level of arterial pressure inasmuch as it was observed to occur following parenteral doses of veratrum insufficient to cause a significant reduction of blood pressure, and to disappear when a continuous hypotension was maintained by prolonged oral administration of the drug. The lack of direct mechanical dependence upon either arterial pressure, glomerular filtration rate or renal plasma flow indicated that the oliguria was due to a specific antidiuretic effect. This conclusion was further substantiated by the regular occurrence of a marked rise in the U/P ratio of inulin or mannitol during the period of depressed urine volume indicating an increased tubular reabsorption of water. In addition, the extraction of PAH from the blood, as measured directly in the renal vein, did not change appreciably during the period of oliguria. These data were interpreted as indicating that the kidneys usually cleared the blood as efficiently after veratrum as they had prior to the induced hypotension but did so at first with the production of a small volume of highly concentrated urine. Therefore, the oliguria induced by the acute administration of veratrum viride did not indicate severe impairment of renal function as has been postulated previously (25), but rather a transient antidiuresis, which disappeared with continued oral treatment.

Whereas mannitol clearances fell and remained low after veratrum viride, inulin clearances fell

initially but usually returned to control values despite a continued reduction in arterial pressure. This discrepancy between the clearances of mannitol and inulin was not surprising in view of the recent evidence that under certain circumstances mannitol may be partially reabsorbed in the tubules (26). The increased tubular reabsorption of water which followed the parenteral injection of veratrum viride might well tend to accelerate the back-diffusion of mannitol, and thereby, to aggravate the discrepancy between the clearances of mannitol and inulin.

Since reflex vasopressor responses and also the vasoconstrictor reactions in the hands and feet all remained intact after veratrum viride, the drug did not appear to block sympathetic vasoconstriction. However, it is interesting that with the fall in blood pressure there was a decrease in blood flow in the sympathectomized extremities of two patients, as contrasted with the increase that occurred in the normally innervated control limbs. The decrease in the sympathectomized limbs may have been the passive result of the reduction in arterial pressure and the increase in the control limbs the result of active sympathetic vasodilatation. In this connection it was of interest also that after veratrum there was an increase in vascular distensibility in normally innervated limbs as contrasted with little or no change in sympathectomized limbs. Thus, while there was no evidence that sympathetic vasoconstrictor reflexes were blocked there was indication that vasodilator responses mediated over the sympathetic nerves might be stimulated. Finally, atropine abolished the bradycardia but did not reverse the hypotensive effect suggesting that the fall in arterial pressure was not dependent upon parasympathetic stimulation.

As a result of these studies one might draw the clinical implication that veratrum viride should be a suitable therapeutic agent in essential hypertension since it can reduce the blood pressure without harmful effects on the heart or kidneys (except when the function of the latter is severely impaired) and, since it leaves intact the vasomotor reflexes, particularly those concerned with assuming the erect posture. However, during actual clinical trial it has caused frequent toxic side reactions, the most prominent of which are nausea and vomiting (5). Work so far indicates that

these reactions may be an integral part of the reflex pattern activated by the drug (2). Therefore, the possibility of isolating or developing a compound of veratrum that will retain the desirable cardiovascular effects of the crude drug and eliminate the toxic side effects does not at present appear to be very promising.

SUMMARY AND CONCLUSIONS

Veratrum viride administered to hypertensive patients produces the following hemodynamic effects:

1. Blood flow through muscular, renal, and hepatic-portal areas usually falls initially and then returns to approximate control levels despite a continued hypotensive response. Full peripheral vasodilatation characteristically follows rather than accompanies the initial reduction in arterial pressure.

2. The output of the compensated heart remains essentially unchanged after arterial pressure is reduced. In congestive heart failure cardiac output may increase and the elevated pulmonary arterial pressure may fall.

3. Atropine abolishes the bradycardia induced by veratrum viride but only partially reverses the hypotension. Since the hypotensive effects do not depend necessarily upon a reduction in either cardiac rate or output, there is little evidence to indicate that the drug is a "cardiac depressant."

4. Effective renal plasma flow decreases only transiently and returns to control values despite a continued reduction in arterial pressure after veratrum. Glomerular filtration rate (inulin) follows a similar pattern in most cases, but occasionally remains below control values.

5. Oliguria occurs after the initial injection of veratrum viride but disappears when the hypotensive effect is continued by repeated oral administration of the drug. The oliguria is not related directly to the arterial pressure, glomerular filtration rate or renal plasma flow, but appears to be due to a specific antidiuretic effect.

6. The discrepancy between inulin and mannitol clearances is confirmed, the mannitol clearances usually remaining lower than those obtained with inulin. This discrepancy seemed to be accentuated during marked antidiuresis suggesting increased tubular reabsorption of mannitol.

7. Veratrum viride does not inhibit sympathetic vasoconstrictor responses as evidenced by the maintenance of vasopressor overshoots, skin temperature levels, vascular reflexes in the digits, the cold pressor response, and postural adaptation.

8. Vascular distensibility in the extremities usually increases, except in sympathectomized limbs, but not necessarily in association with alterations in arterial pressure, blood flow or peripheral resistance.

9. During the period of reduced arterial pressure, blood flow and vascular distensibility in sympathectomized as compared with normally innervated extremities do not increase, indicating that part of the vascular response in the limbs may be mediated over sympathetic nervous pathways possibly through sympathetic vasodilator fibers.

10. Epinephrine and ephedrine reverse the hypotensive effects of veratrum.

11. The evidence cited indicates that veratrum can produce an integrated hypotensive response which is not associated with toxic effects on either the heart or the kidneys.

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SOME OBSERVATIONS ON THE BALLISTOCARDIOGRAPHIC PATTERN, WITH SPECIAL REFERENCE TO THE H AND K WAVES¹

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The origin of certain portions of the ballistocardiographic pattern has been discussed only briefly in the physiological literature. Starr and his associates (1) have given letter designations to the salient peaks and troughs and have discussed in some degree the origin of the I and J waves. Hamilton and his coworkers (2, 3) have suggested that the pattern may be due in part to the oscillations of the blood mass within the larger portions of the arterial tree. It is the purpose of the work being presented here to consider the results of some simple experiments which help elucidate in broad outline the significance of several parts of the ballistocardiograph pattern.

Two sources of information have been tapped in this study, to wit, model experiments and clinical observations. The ballistocardiograph used in this investigation was not the instrument constructed by the workers mentioned above but was the low frequency, critically damped instrument designed by Nickerson and Curtis (4). The heart model built for the study of the ventricular contribution to the ballistic pattern was a simple one-chamber device. This model ignores the asynchronism which sometimes appears between the ejection of the left and right ventricles (5, 6) and also ignores the auricular contribution to the impacts, a problem which will be dealt with in a different fashion.

The construction of the model heart is demonstrated in Figure 1. It consists of a closed elastic system of bulbs and tubing filled with water. The dimensions of the tubing approximate those of the aortic tree of an adult human. One elastic bulb is contained in a glass pressure bottle so that the force on this bulb may be raised to any suitable level. The tubing used in this system is of rubber and the bulbs are rubber balloons of various

thicknesses. This bottle has sufficient volume that the pressure changes in it during ejection are small. In operating this system, water to any desired amount (30-100 cc.) is squeezed back into the bulb in the pressure bottle at a time when the pressure bottle is open to the atmosphere. The exit tube from this bulb is then clamped shut and the pressure in the bottle raised to a measured level (40-120 mm. Hg). If now the quick-acting clamp is suddenly released by burning through a restraining thread the water in the heart bulb will be forced into the outer portions of the circulatory system corresponding to the aortic tree. This movement of fluid produces a reaction equivalent to systolic ejection. By varying the filling of the heart bulb and also the pressure in the bottle it is possible to control both the stroke volume and the force of ejection.

The normal human ballistocardiogram is illustrated in Figure 2 in which the H, I, J, K and L waves of Starr's notation are identified. It is

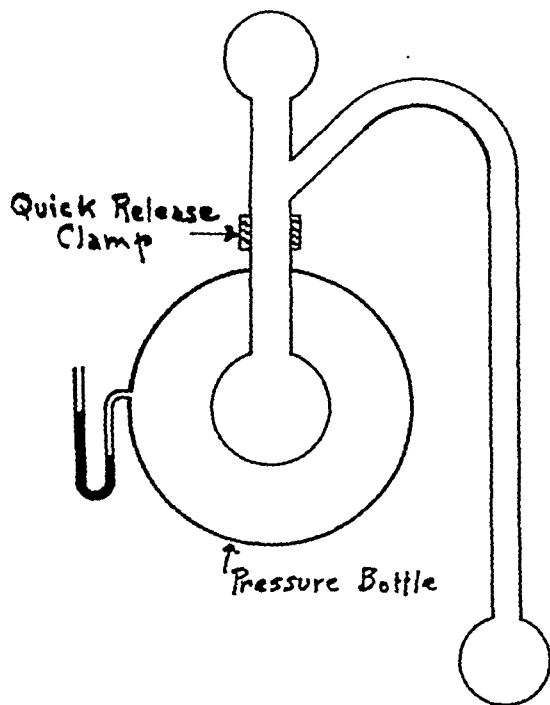


FIG. 1. DIAGRAM OF THE MODEL HEART

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FIG. 2. BALLISTOCARDIOGRAMS ON NORMAL SUBJECTS

the general view that the I-wave is produced by the footward recoil of the body and ballistic bed as a result of the headward movement of the blood at the time of its ejection from the left and right ventricles during systole. After the commencement of this footward movement, the blood, which is travelling headward, begins to fill up the aortic tree, and so slows in its course. At the same time some portion of the blood actually reverses its direction by passage around the aortic arch. These occurrences result in a large headward recoil which is sufficient to neutralize and reverse the footward movement, thus driving the ballistic system headward to produce the J-wave. Following this impact the system reaches its maximum headward deflection and since it is critically damped might be expected to return to the baseline position without overshooting. However, the blood rushing down the descending aorta does

not continue with undiminished speed but in the lower abdominal region is slowed in its footward rush, thus producing a footward thrust. This thrust is on most occasions so timed that it drives the ballistic system footward across the baseline, thus generating the K-wave. Following the K-wave there is frequently a return to the baseline. However, in many cases a subsequent headward wave, the L-wave, appears which is likely due to a reflected wave of blood travelling first up and then down the long column of the descending aorta.

That this normal pattern can be reproduced by the model heart's ejection is shown by Figure 3. Records 1 and 2 were produced by essentially the same stroke volume and ejection pressure and differ only in the greater length of the descending aorta in Record 1. The slope of the I-J line in both records is the same. Hence the stroke

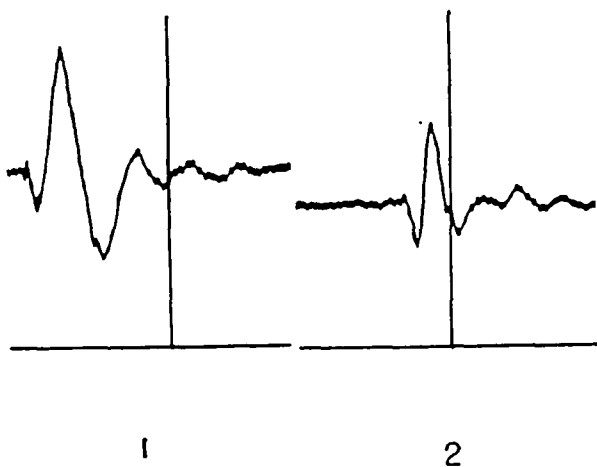


FIG. 3. BALLISTOCARDIOGRAMS FROM THE MODEL HEART DEMONSTRATING VARIATION IN THE K-WAVE

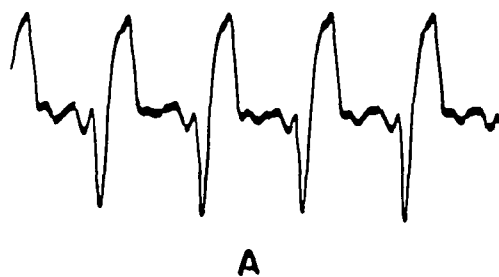
In part 1 the descending aorta is long while in part 2 it is short.

volumes are equal. However, the relative depths of the K-waves are quite different. Further observation has shown that variation in the length

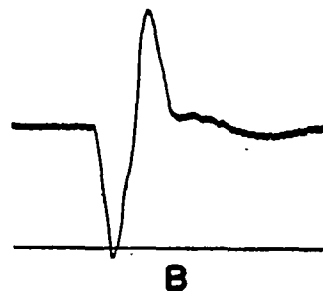
of the descending aorta is singularly effective in changing the K-wave and appears to be one of the chief factors determining the depth of this wave in the normal human ballistocardiogram.

Further shortening of the descending aorta or constriction of this vessel so that the blood following this course does not have a clear path below the aortic arch produces complete disappearance of the K-wave. This type of pattern is demonstrated in Figure 4A by an actual coarctation of the aorta and in Figure 4B by a coarctation at the arch of the aorta in the model experiment. Similarly in all cases of coarctation of the aorta in which it has been possible to obtain low frequency, critically damped ballistocardiograms, diminution or disappearance of the K-wave is found. It is to be noted that this absence of the K-wave is not observed when a conventional high frequency ballistocardiograph is used (see Figure 5). It is proper to state at this point that although all patterns of coarctations observed with

COARCTATION



PRE OPERATIVE



POST OPERATIVE

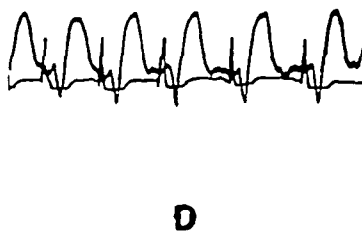


FIG. 4

- A. Coarctation of the aorta in a patient.
 B. Coarctation of the aorta in the model at the beginning of the descending aorta.
 C and D. Pre- and post-operative records on a patient with coarctation of the aorta. The surgical procedure was the anastomosis of the subclavian artery to the descending aorta.

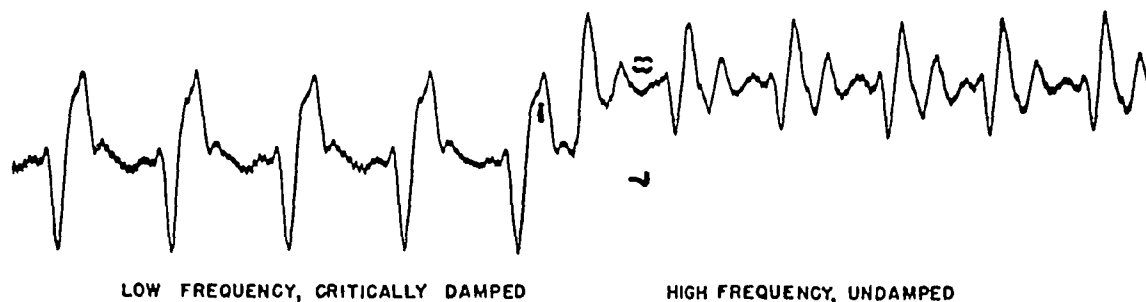


FIG. 5. RECORD ON A PATIENT WITH COARCTATION OF THE AORTA TAKEN WITH A LOW FREQUENCY, CRITICALLY DAMPED BALLISTOCARDIOGRAPH AND WITH A HIGH FREQUENCY, UNDAMPED BALLISTOCARDIOGRAPH

the low frequency, critically damped ballistocardiograph show this reduction of the K-wave, nevertheless a few records are seen where the K-wave is diminished without aortic coarctation being present. From analogy with the model experiments it is believed that this result appears when the effective free lengths of the major portions of the headward regions of the aortic tree and the descending aorta are essentially equal. To date only one patient with coarctation of the aorta on whom surgical treatment has been performed has had pre- and post-operative ballistic records taken. The results obtained are shown in Figures 4C and 4D. In this case the anastomosis accomplished was of the subclavian to the aorta, a procedure by which the circulation to the extremities was improved. As a result of this particular anastomosis the momentum of the blood in the descending aorta was still impeded in a region high in the thorax rather than low in the abdominal cavity as is normal. For this reason, although the movements producing the K-wave post-operatively are more accentuated than in the pre-operative pattern, the results, however, are not as completely normal as would be expected had the anastomosis been of the aorta on itself.

Another point of evidence for the development of the K-wave arises in the change of pattern on the production of a reactive hyperemia of the lower extremities. This reaction appears if the circulation to the lower limbs is occluded by pressure cuffs applied to the thighs with pressures above the systolic level for a period of 15 minutes and then released. The resulting increased oxygen demands of the tissues result in an increased blood flow to the affected parts. The changes in the ballistocardiogram are shown in Figure 6. There are two chief changes, first, an increase in

the slope of the I to J movement, thus indicating an increase in stroke volume (7, 8), and second, a decrease in the relative depth of the K-wave. This diminution of the K-wave occurs since the retardation of the blood velocity at the lower end of the descending aorta is not so great as normal, more of the blood flowing directly into the wider open vascular bed of the lower extremities. Thus the impulse producing the K-wave is less than before.

The effect of reducing the stroke volume in the model heart produces some variations in the size of the pattern. These are shown in Figure 7 where are illustrated the types of pattern found in a patient in shock. These general variations were found in all our shock cases. In this particular case the first record was made before treatment while the second and third records were made after treatment with albumin and with saline respectively. The values of the stroke volumes from the Fick data were successively 26, 37, and



REACTIVE HYPEREMIA



FIG. 6. CHANGE IN THE FORM OF THE PATTERN ON THE OCCURRENCE OF A REACTIVE HYPEREMIA OF THE LOWER LIMBS

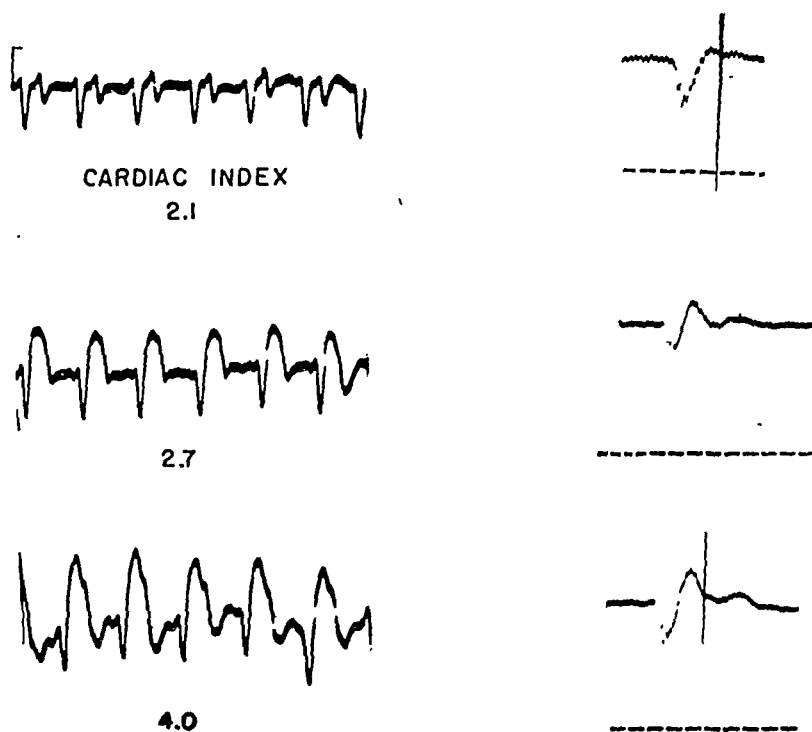


FIG. 7. SUCCESSIVE BALLISTOCARDIOGRAMS ON A PATIENT IN SHOCK (CARDIAC INDEX BY THE FICK = 2.1), AFTER TREATMENT WITH ALBUMIN (INDEX = 2.7) AND AFTER FURTHER TREATMENT WITH SALINE (INDEX = 4.0)

The accompanying records are of the model heart with increasing stroke volume.

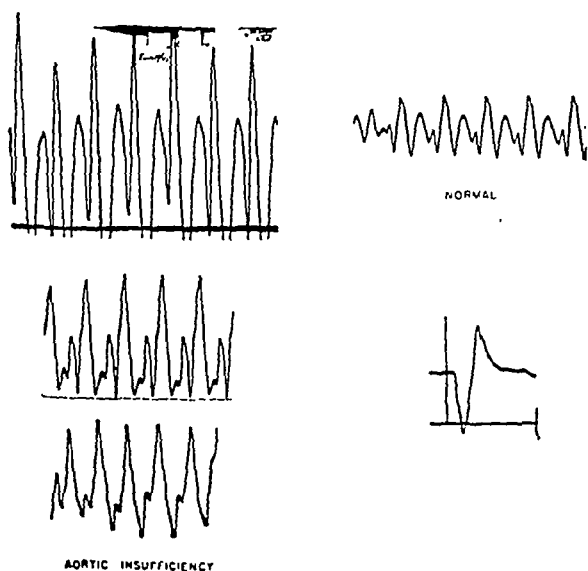


FIG. 8. BALLISTOCARDIOGRAMS ON THREE PATIENTS WITH AORTIC INSUFFICIENCY, ON A NORMAL SUBJECT AND ON THE MODEL HEART IN A SITUATION WHERE A LARGE STROKE IS EJECTED INTO A FLACCID AORTIC TREE

55 ml. The most significant parallelism from the patient's records and from the records of the model heart is the manner in which the J-wave increases more markedly than the I-wave as the stroke volume returns toward normal. The low J-wave may possibly be a distinguishing mark of a stroke volume below normal.

The other extreme of an unusually large cardiac stroke volume produces also a characteristic pattern, Figure 8. The situation demonstrated is that of aortic insufficiency where the excessively large stroke volume is partly returned to the heart through the faulty aortic valve during diastole. The most striking features of these records are their unusual size (*i.e.*, the I-J slope is extremely steep) under basal conditions as compared with normal records, and the appearance in many records of a slurring in the rising limb of the J-wave. The latter feature was reproducible in the model heart as is also shown in Figure 8. This particular pattern occurs when

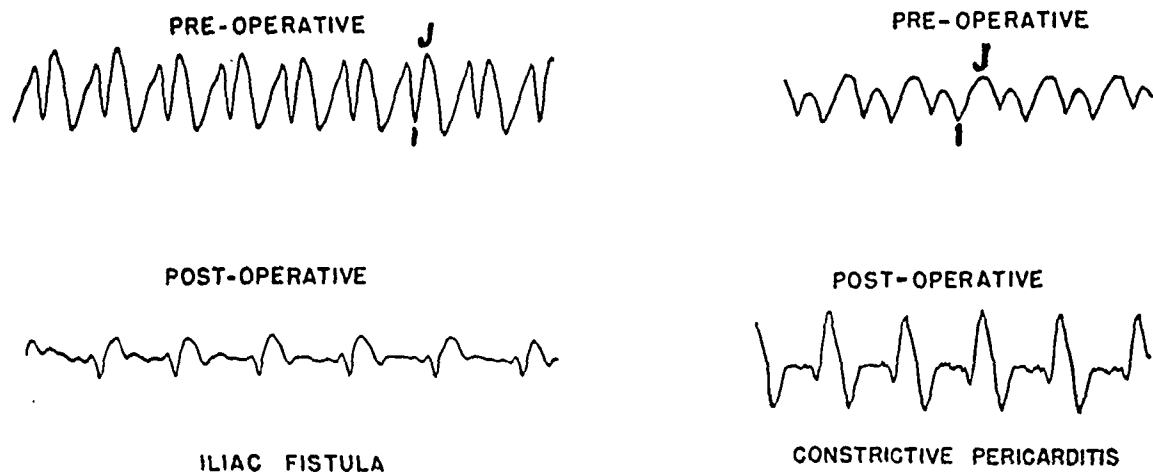


FIG. 9

the stroke volume of the model is large and the bulb at the head end of the model is of unusually stretchable rubber.

In Figure 9 are shown several patterns which are dramatically abnormal. The first is a pre-operative iliac arteriovenous fistula in which the fistula carried a large flow of blood. The pattern presents a sequence of footward and headward movements of almost equal size. A possible explanation of this pattern may be that it is the result of a series of impacts due successively to the footward thrust of the ventricular ejection, the headward thrust at the turn of the arch of the aorta, etc., the large footward impact caused by blood changing its direction in passing through the iliac fistula and finally the return to the baseline in time for the cycle to begin again. The post-operative pattern after removal of the fistula shows a slowing of the heart, a decrease in the stroke volume and a restoration of the pattern to a more normal form. In particular the influence of the fistula upon the K-wave should be noted. The second illustration in this figure is a case of constrictive pericarditis. The pre-operative pattern shows a small I-J slope which indicates a stroke volume well below the normal basal value. The post-operative pattern is remarkably improved toward normal and has a normal value of the basal cardiac index.

Figure 10 illustrates changes in the pattern which appear as a result of irregular rhythms of the heart. Part 10 A presents the pattern observed in sinus arrhythmia and shows that the variations are largely in the size of the pattern, the longer filling period being followed by a

steeper I-J slope indicating a larger stroke volume, and the shorter intervals between beats being followed by smaller patterns and therefore

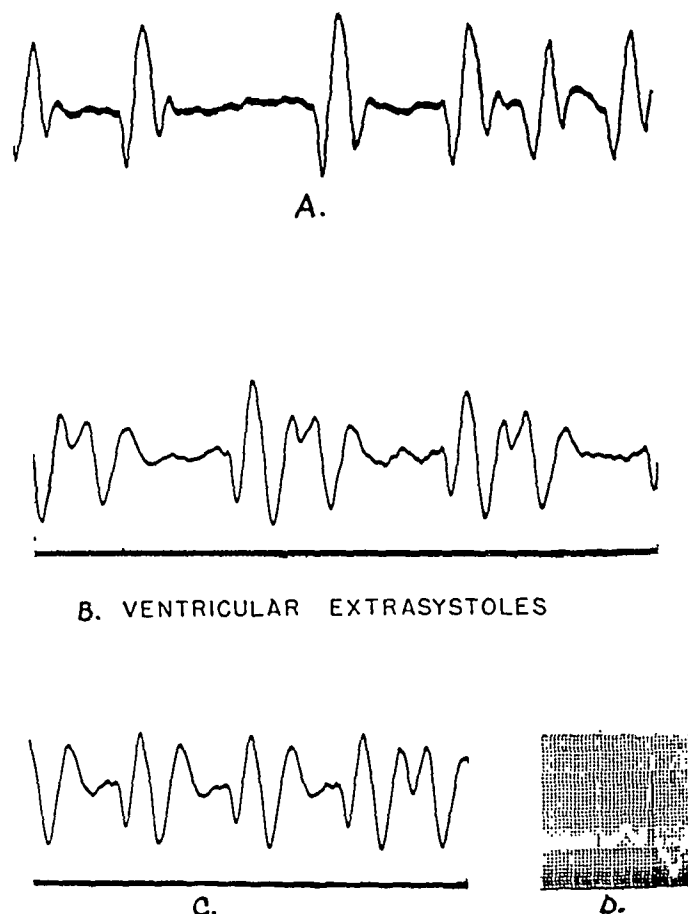


FIG. 10

- A. Irregular beats originating in the sinus node.
- B. Extrasystoles of ventricular origin.
- C. Same patient (B) when extrasystoles are not occurring.
- D. ECG on patient in B and C showing normal and abnormal ventricular patterns.

smaller stroke volumes. Part 10 B illustrates the result of a close succession of beats. The pattern of the premature beat begins before the termination of the pattern of the preceding normal beat and permits only a short filling time. Since this premature beat is not observable in the radial pulse, it is quite possible that the ballistic movement accompanying this beat is not the result of the impact caused by an ejection of blood but is rather an indication of the contribution of the movement of the heart mass to the pattern. Part 10 C shows the ballistic pattern on the same subject at a time when these premature beats are not occurring.

So far in this discussion the presence of the H-wave has been ignored and the movements of the ballistic system considered as beginning with a footward excursion starting at the peak of the H-wave. However, as pointed out by other workers (3), this is probably not correct. For

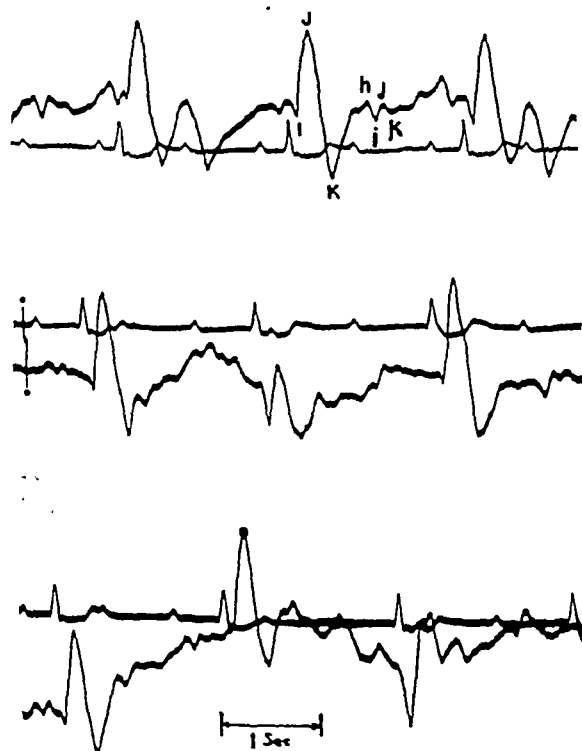


FIG. 11. BALLISTOCARDIOGRAM OF A PATIENT WITH COMPLETE HEART BLOCK

The auricular complex *h, i, j, k* is shown as well as the purely ventricular complex *I, J, K, . . .* Where overlapping of the two complexes occurs, the usual H-wave of the ballistocardiogram appears.

TABLE I

No.	Ph	Pi	Pj	Pk	PR	QH	QI	QJ
1	0.11	0.15	0.24	0.31				
2	0.11	0.15	0.24	0.31	0.20	0.09	0.13	0.23
3	0.11	0.15	0.25	0.33				
4		0.13	0.25	0.29	0.31	0.07	0.14	0.24
5					0.35	0.07	0.13	0.24
6	0.11	0.15	0.25	0.31	0.40	0.07	0.13	0.24
7	0.11	0.16	0.23	0.31	0.54	0.065	0.14	0.23
8	0.12	0.17	0.23	0.35	0.51	0.065	0.14	0.25
9	0.15	0.20	0.27	0.37	0.40	0.07	0.15	0.26
10	0.14	0.19	0.27	0.37	0.64	0.07	0.16	0.25
11					0.20	0.10	0.19	0.25
12	0.11	0.17	0.23	0.31	0.73	0.08		
13	0.09	0.16	0.25	0.35	0.27	0.08	0.13	0.26
14					0.36	0.07	0.13	0.25
15	0.09	0.16	0.24	0.30	0.49	0.07	0.13	0.24
16		0.16	0.24	0.29	0.60	0.065	0.14	0.25
17					0.23	0.085	0.14	0.25
18		0.13	0.23	0.30	0.34	0.08	0.13	0.24
19		0.15	0.24	0.31	0.51	0.08	0.13	0.25
20					0.67	0.07	0.12	0.25
21	0.08	0.13	0.23		0.21	0.08	0.13	0.24
22					0.065	0.07	0.14	0.25
23					0.19	0.09	0.14	0.26
24		0.14	0.23	0.30	0.32	0.08	0.13	0.24
25		0.15	0.24	0.31				
26					0.07	0.07	0.13	0.25
27					0.13	0.09	0.14	0.26
28	0.07	0.16	0.24	0.29	0.41	0.07	0.13	0.24
29	0.08	0.15	0.24	0.29	0.70	0.065	0.13	0.26
30	0.09	0.15	0.24	0.31	0.40	0.075	0.14	0.23
31	0.09	0.15	0.24	0.31				
mean	0.104	0.155	0.242	0.315			0.137	0.247

P = beginning of P wave of the electrocardiogram.

Q = beginning of the QRS complex of the electrocardiogram.

h, i, j, k are successive peaks in the auricular complex of the ballistocardiogram.

H, I, J are successive peaks in the normal ballistocardiogram.

PR = the usual interval defined as the time from the beginning of the P wave to the beginning of the QRS complex of the electrocardiogram.

The table above lists the time in seconds between the points so defined.

example, the time interval from the beginning of the QRS complex of the electrocardiogram to the peak of the H-wave is about 0.100 second whereas the time from the QRS complex to the beginning of the ventricular ejection is about 0.0700–0.0800 second (9). Because of this discrepancy, it has been suggested that the I-wave of the ballistocardiogram does not follow the ventricular ejection but rather is associated with some other circulatory event. Of course the possibility exists that the ballistic movements of the

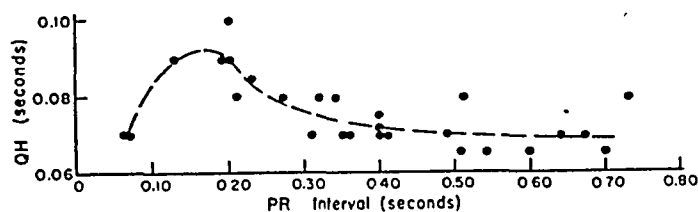


FIG. 12

QH, the time from the beginning of the QRS complex to the beginning of the footward movement producing the I-wave, is plotted against the PR interval, which is taken as an index of the amount of overlapping of the auricular and ventricular ballistic complexes, in a patient with complete heart block.

auricular ejection may act in such a way as to obscure the beginning of the ventricular ejection phase. In order to check this explanation, simultaneous recordings of the ballistocardiogram and the electrocardiogram have been made on several patients having heart block. Figure 11 shows some of the complexes recorded on a patient with complete heart block. The data on the auricular and ventricular complexes are given in Table I. The auricular complex is small and appears to consist successively of a headward (h), a footward (i) and a headward (j) wave of which the average time intervals from the beginning of the P of the electrocardiogram to the peaks of these waves are, respectively, $P_h = 0.10$ second, $P_i = 0.16$ second, and $P_j = 0.24$ second. The purely ventricular complex appears to start directly footward from the baseline with no H-wave in evidence. For this pure ventricular pattern the time from the beginning of the QRS complex to the beginning of the footward movement which produces the I-wave is about 0.070 second, a value which is close to the accepted value for the time from the QRS to the commencement of ventricular ejection. However, this pure ventricular complex is seen only when the PR interval is sufficiently long that the auricular and ventricular complexes do not overlap. This overlap is a function of the PR interval and its effect is illustrated in Figure 12. In this figure the

QH interval (H being the point where the ballistic movement first turns footward) is plotted against the PR interval. When the PR interval is long, the QH interval is about 0.070 second. However, when the PR interval lies within normal range, *i.e.*, from 0.10 to 0.20 second, the QH interval is about 0.090 second. This observation suggests that the auricular j-wave is an important factor in producing the H-wave and in delaying the footward movement of the I-wave. Similarly when the PR interval has a value less than normal the QH interval decreases again. In this case it may be that the footward movement is accentuated by the footward i-wave of the auricular complex. This view that the auricular stroke contributes markedly to the H-wave of the ballistocardiogram is supported by the type of ballistic pattern observed in auricular fibrillation. In this case, see Figure 13, the QH interval is relatively short, 0.060–0.70 second. This value would be expected if the auricular beat was ineffective in moving fluid so that the ballistic movement is almost purely ventricular in origin. As a result of these studies it is concluded that in the ballistocardiograms of normal subjects the auricular impact may act to produce the H-wave and in this action mask the onset of the I-wave.

SUMMARY

In this paper have been presented ballistocardiograph records of clinical cases and of the ejection behavior of a simple model heart. Evidence to support the origin of the K-wave is found in records of coarctation of the aorta and of reactive hyperemia. Characteristic patterns of pre- and post-operative cases of coarctation of the aorta, of constrictive pericarditis and of an iliac arteriovenous fistula are also presented here. Further are shown the form of records with extremely large and small stroke volumes as found respectively in the special conditions of aortic insufficiency and of shock.

Data have been presented from the observa-



FIG. 13. BALLISTOCARDIOGRAM ON A PATIENT WITH AURICULAR FIBRILLATION

tions made on cases in heart block. This material illustrates the contribution of the auricular ejection to the ballistocardiogram and shows that in cases with normal PR intervals the auricular beat may produce an apparent delay in the development of the I-wave, a delay attributed by some workers to faulty behavior of the ballistocardiograph.

ACKNOWLEDGMENT

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TRANSFERS OF POTASSIUM IN RENAL INSUFFICIENCY¹

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Elevation in concentration of serum potassium in patients with renal insufficiency has been observed many times but has been considered to be a relatively rare occurrence (1-3). Reports of abnormally low levels of serum potassium in this disorder have been extremely infrequent (4-8). Nevertheless, the occasional finding in such patients of disturbances in the exchanges of potassium, has directed interest toward the clinical significance of these abnormalities.

Winkler, Hoff and Smith were the first to recognize that the toxicity of a high concentration of potassium in serum and extracellular fluid is due to its effect on the conduction system of the heart. In animals rendered anuric by nephrectomy or by ureteral ligation, death regularly resulted from extracellular accumulation, to cardiotoxic levels, of potassium released from tissue cells (9, 10). Such accumulation was speeded by the administration of potassium (10) and retarded by depletion of potassium prior to the establishment of the anuria (11). The potassium content of tissue cells in anuric animals has been shown to vary (12), but experiments in cats indicated that the lethal factor was the elevation of extracellular potassium concentration, not a change in the potassium of the cells (13).

Intolerance of patients with severe renal disease to administered potassium has been recognized in the past (14) and was clearly demonstrated by Winkler, Hoff, and Smith (15). Slight elevations of the serum concentration were found in five of 12 patients studied, but no cardiotoxicity was revealed in electrocardiographic tracings. Since then definite, and possibly fatal, changes in cardiac conduction have been reported in such

patients with high concentrations of serum potassium (16-18).

It is evident, therefore, that in renal insufficiency the concentration of potassium in extracellular fluid may rise, that a number of variables influence the occurrence and rate of such a rise, and that the rise may threaten the life of the patient. Death may also occur in the presence of a normal or low serum concentration and be due to causes quite unrelated to disturbances of potassium metabolism.

The study reported here is an attempt to determine more exactly the frequency of, and the physiological factors involved in, abnormal exchanges of potassium in patients with severe renal insufficiency. The data are presented from the standpoint that abnormality of the concentration of serum potassium in cases of renal insufficiency with reduced glomerular filtration is a function of extracellular fluid volume, intake and output of potassium, and its distribution between the extra- and intracellular phases.

EXPERIMENTAL MATERIAL AND PROCEDURE

During a period of nine months 51 patients had concentrations of blood non-protein nitrogen greater than 100 mg. per cent; in 26 of these patients one or more measurements were made of the concentration of serum potassium. These 26 patients constitute the clinical ma-

TABLE I

Distribution of abnormal concentrations of serum potassium in patients with renal insufficiency according to type of renal disease

Diagnosis	Number of cases	Concentration of serum potassium in m.eq. per liter			
		<3.5	3.5-5.5	5.6-6.4	>6.4
Nephrosclerosis	9	2	6	2	5
Lower nephron nephrosis	5	1	2	1	1
Pyelonephritis	3	0	2	0	1
Glomerulonephritis	2	0	1	2	1
Other	7	1	4	3	3
Total	26	4	15	8	11

¹ Aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

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TABLE II

*Patients with renal insufficiency on whom balance studies were made:
External transfers of water, electrolytes, and nitrogen*

Pt.††	Period	Intake						Urine					Balance*			
		H ₂ O	Cl	Na	K	N	CHO	Vol.	Cl	Na	K	N	Cl	Na	K	N
	<i>dates</i>	<i>cc.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>grams</i>	<i>grams</i>	<i>cc.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>grams</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>m.eq.</i>	<i>grams</i>
P. McG.	12/24-25	1275†	265	263	15	1.8	45	110	2	3	6	0.6	+ 255	+252	+ 10	+ 1.2
A. M.	1/20-21	7000	423	485	4	12.4	275	±0					+ 420	+ 481	+ 4	+12.4
	1/21-22	3250	102	380	1	2.8	225	270	14	16	9	0.9	+ 86	+ 361	- 8	+ 1.9
	1/22-23	4250	541	765	1	2.8	50	90	11	14	4	0.6	+ 528	+ 748	- 3	+ 2.2
	1/23-24	4550	387	611	1	2.8	150	100	8	10	2	0.4	+ 377	+ 598	- 1	+ 2.4
C. K.	1/20-21	3190	96	378	2	5.6	180	740	22	26	25	3.6	+ 70	+ 346	- 23	+ 2.0
W. M.	12/13-16	8000†	77	113			500	597	28	38	11	2.1	+ 44	+ 66	- 11	- 2.1
	12/16-19	4500†					250	2805	180	224	43	9.3	- 184	- 231	- 43	- 9.3
W. S.	2/11-14	7620	1474	1605	2	5.6	320	2580	26	36	102	17.4	+1442	+1560	-100	-11.8
	2/14-16	6170†	140	185	29	4.5	155	885	33	32	33	5.9	+ 105	+ 161	- 4	- 1.4
	2/16-17	2500	36	0	0	0	200	375	6	5	17	2.6	+ 28	- 8	- 17	- 2.6
	2/17-18	1500	0	0	0	0	75	200	6	5	17	2.6	+ 8	- 8	- 17	- 2.6
	2/18-19	1500	0	0	0	0	150	986	15	11	47	6.5	- 17	- 14	- 47	- 6.5
P. DeB.	2/28-3/1	1800†	17	48	42	21.1	160	350§	15	14	18	5.9	- 1	+ 30	+ 24	+15.2
	3/1-2	5600†	12	221	16	24.2	170	20	—	—	—	—	+ 7	+ 213	+ 16	+24.2
	3/2-3	700†	274	307	17	17.1	70	20	—	—	—	—	+ 266	+ 295	+ 17	+17.1
	3/3-4	1900†	8	40	20	14.1	110	0					+ 4	+ 34	+ 20	+14.1
	3/4-5	2000†	12	48	43	21.6	200	0					+ 8	+ 42	+ 43	+21.6
	3/5-6	1170†	24	58	16	8.2	120	25	—	—	1	—	+ 21	+ 53	+ 15	+ 8.2
L. T.	1/6-7	2000	25	35	2	2.8	75	95	4	4	3	0.7	- 57**	- 18	- 9	+ 0.5
R. S.	11/4-8	1000	0	0	0	0	0	0								
J. P.	4/14-15	3000†	55	61	15	2.9	150	0					+ 53	+ 58	+ 15	+ 2.9
	4/15-16	2440†	196	190	53	19.0	211	250	9		9	2.3	+ 182	+ 44	+ 44	+16.7
	4/16-16	2000†	6	6	2	0.1	150	0					+ 1	- 1	+ 2	+ 0.1
F. S.	3/20-21	2000	96	127	2	6.2	100	2200	87	148	18	4.4	+ 7	- 24	- 16	+ 1.8
	3/21-22	2750	212	243	2	6.2	75	1400	58	89	10	3.1	+ 152	+ 151	- 8	+ 3.1
	3/22-23	2400	253	204	82	6.2	100	1550	84	104	25	6.1	+ 162	+ 89	+ 57	+ 0.1

* Balances of Cl and Na corrected for small quantities lost in serum drawn for analysis.

† Includes small quantities taken orally, the contents of which were estimated from Sherman's tables (43).

‡ Includes oral liquids in small quantities, which probably contained only small amounts of electrolyte and nitrogen.

§ Includes stool, 75 cc. urine, and 100 cc. vomitus.

** Balances include 550 cc. of vomitus, which contained 75 m.eq. Cl, 45 m.eq. of Na, 8 m.eq. of K, and 1.6 grams of N.

†† Diagnoses are indicated in Table IV.

terial for the survey. They are a selected group in that potassium determinations were more frequently done in patients who were oliguric and in patients who were on the medical service. The concentration of serum potassium was followed with varying frequency and in variable proximity to the time of death. There were many types of kidney disorders responsible for the renal insufficiency in these patients; the diagnoses were made from clinical data, and from necropsy findings when available.

Ten of the 26 patients were studied more intensively. During periods which varied from 12 hours to eight days, exchanges of chloride, sodium, potassium, and nitrogen were measured and the transfers of potassium between the various phases of body fluid were calculated. In some, the volume of distribution and clearance of manni-

tol were determined, to approximate the volume of extracellular fluid and the glomerular filtration rate (19). Electrocardiograms were taken simultaneously with many of the determinations of serum potassium.

The chemical methods and mode of calculation have been detailed in previous communications (20-22).

RESULTS

The analytical and derived data are presented in Tables I-V and in Figures 1 and 2.

The concentration of serum potassium was greater than 5.5 m.eq. per liter in more than one-half the cases; abnormal concentrations were not exclusively associated with any one kind of

renal lesion (Tables I, III). In 11 of the 26 patients it was greater than 6.4 m.eq. per liter, the concentration just above which electrocardiographic changes attributable to the concentration of serum potassium sometimes appeared (23). Abnormally low concentrations were much less frequent, being found in only 4 of the 26 patients.

In most of the patients the concentration of serum potassium rose as the renal insufficiency progressed (Table III). Distinct decline of serum potassium, as death approached, occurred in at least one patient. In 18 patients concentrations of serum potassium were measured less than 24 hours before death; of these 12 were elevated, four were normal, and two were low. In certain patients changes in the concentrations of blood non-protein nitrogen and of serum potassium varied in similar directions (Table III), but for the group as a whole there was a wide variation

between any given level of potassium and any given level of non-protein nitrogen.

Oliguria or anuria appeared to be a prerequisite to elevation of the serum concentration. None of the patients with concentrations of serum potassium above 6.6 m.eq. per liter produced more than 250 cc. of urine during the preceding 24 hours (Figure 1). On the other hand, periods of anuria or oliguria were not necessarily followed by elevation of serum potassium.

The factors which determine a given concentration of potassium in serum are the volume of extracellular fluid and the amount of extracellular potassium which results from exchanges with the cells on the one hand and the external environment on the other. These exchanges were calculated in detail from the balance data obtained in 10 patients (Tables II and III), and are summarized for nine patients in terms of average

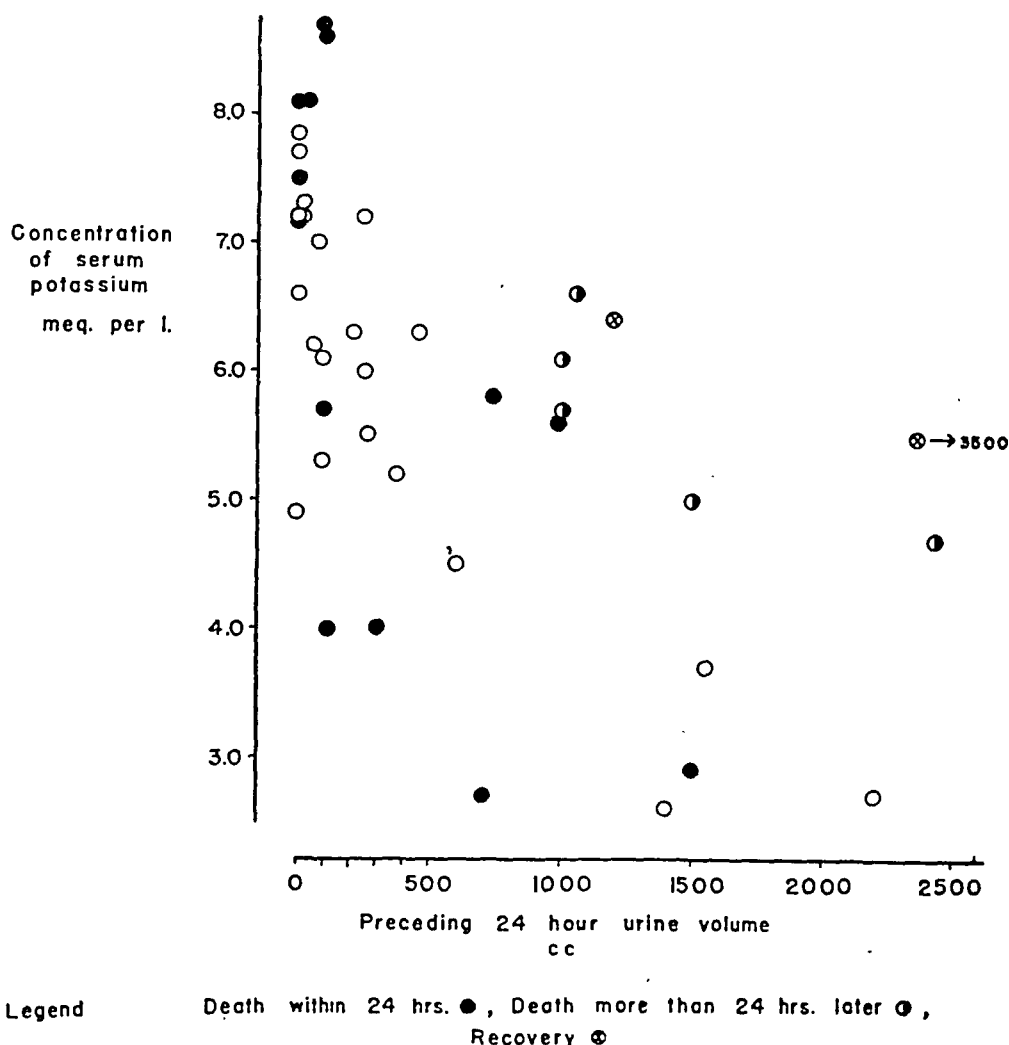


FIG. 1. THE RELATION OF THE CONCENTRATION OF SERUM POTASSIUM TO URINE VOLUME

daily values in Table IV. In most of the patients the volume of extracellular fluid expanded. Although the rate of expansion varied considerably, it may have ameliorated any rise in potassium concentration which would have resulted from accumulation of the ion in that fluid phase. In three of the five patients in whom the final concentration of serum potassium was greater than 7.4 m.eq. per liter, the small amount of potassium ingested in food exceeded the urinary output, and the difference, or positive balance, was equal to part or all of the increase in extracellular potassium. In all of the four patients whose final concentrations of serum potassium were less than 6.5 m.eq. per liter, the intake of potassium was negligible and the patients were in negative balance with respect to the ion. A net transfer of potassium from cellular to extracellular phase took place in most of the cases; only in two patients did the cells take up potassium. In four cases in which the ion accumulated in the extracellular phase, the cellular decrement was at least as great as the extracellular increment. If the transfers of intracellular potassium are arbitrarily subdivided into those which move with nitrogen according to cell anabolism or catabolism, and those which enter or leave intact cells, the data indicate that in only two cases did part or all of the potassium from catabolized cells enter intact cells. The storage of potassium by the cells did not appear to be directly correlated with the in-

take of carbohydrate or insulin (Tables II, IV).

Electrocardiograms were obtained from four patients when the serum potassium was greater than 7.4 m.eq. per liter (23). All showed toxic effects of potassium. These patients died suddenly. In one patient, P. DeB., an electrocardiogram was obtained within a few seconds of death. This tracing showed widespread disorganization and prolongation of the QRS complex. Although the concentration of serum potassium was 8.1 m.eq. per liter, it is impossible to assert that it was responsible for the cardiac arrest, as other factors probably play a role in the death of these patients. Other signs and symptoms that have been reported as evidence of potassium intoxication were not observed.

The primary disturbance in these patients was the inability of the kidney to excrete potassium. In comparison with previously reported subjects with normal kidneys, and whose intake of potassium was low (22), the rate of excretion of potassium was lower and the urine:plasma concentration ratio (U/P) was about the same (Figure 2). The rate of excretion and the concentration ratio of potassium were reduced in patients with renal insufficiency when contrasted to normal subjects on an intake high in potassium. Nevertheless, even this limited excretory ability sufficed to prevent an accumulation of potassium in the extracellular fluid when the intake of potas-

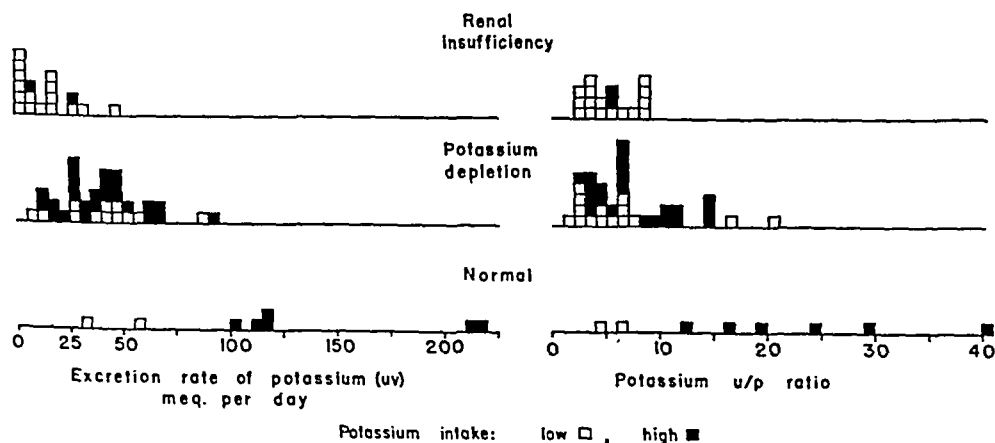


FIG. 2. THE EXCRETION RATE AND CONCENTRATION RATIO OF POTASSIUM IN SUBJECTS WITH RENAL INSUFFICIENCY AND IN THOSE WITH NORMAL KIDNEYS WITH AND WITHOUT POTASSIUM DEPLETION

Each square indicates one subject period.

TABLE III
Patients with renal insufficiency on whom balance studies were made. Analyses of blood and serum and changes in the distribution of sodium and potassium between the phases of body fluid

TABLE III Analyses of blood and serum and changes in the distribution of sodium and potassium between the phases of body fluid																				
Pt.	Diagnosis	Time	Weight	Blood NPN	Serum										Change in			Change in cellular		
					CO ₂	Cl	Na	K	H ₂ O*	Total pro- tein	Ca	P	b _N †	Extra- cellular volume‡	Cell Na	Extra- cellu- lar K	Total	With N	In excess of N	
			kg.	mg. per 100 cc.	m.eq. per liter	m.eq. per liter	m.eq. per liter	m.eq. per liter	grams per liter	grams per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	grams	liters (18.7)§	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	
P. McG.	Lower nephron nephrosis	12/24 9 a.m. 12/24 7:15 p.m.	61.8	186	13.0	76.7	115.9	7.2	935	5.22	6.15	8.68	— 5.7	19.5	+ 61	+33	-23	-13	- 10	
A. M.	Nephrosclerosis	1/20 1/21 1/22 1/23 1/24		235 259 239 243 245	7.0 4.5 8.0 10.7 10.5	95.2 94.5 90.6 95.4 94.1	125.0 128.2 135.0 137.9 131.4	6.7 6.6 5.5 5.3 5.7	943 943 940 937 935	5.61	8.56	13.6	— 0.6 +13.1 — 0.2 + 1.2	18.9 23.0 24.7 28.3 (32.2)§	-109 -39 +158 +258	+25 -16 +14 +34	-21 + 8 -17 -35	- 2 +31 0 + 3	— 19 -23 -17 - 38	
C. K.	Intercapillary glomerulosclerosis	1/20 1/21	60.0	187 204	7.7 14.2	95.4 93.7	132.4 134.9	6.6 5.8	949	4.85	8.22	13.6	— 5.1	(12.0)	+194	- 4	-19	-12	- 7	
W. M.	Lower nephron nephrosis	12/13 12/16 12/19	86.3 87.2 83.0	190 198 196	19.5 18.0 18.1	85.5 83.7 84.5	133.5 131.9 133.8	6.2 6.0 6.4	941	5.91	9.20	7.1	- 7.1 — 8.1	(24.8) 25.9 23.7	- 14 - 11	+ 1 - 3	-12 -40	-17 -19	+ 5 -21	
W. S.	Nephrosclerosis	2/11 2/14 2/16 2/17 2/18 2/19	70.0	215 212 249 271 282 299	20.4 16.3 16.7 26.6 13.5 24.8	83.2 95.0 90.0 88.0 88.5 92.8	122.7 143.2 140.5 134.9 133.5 124.4	3.9 4.2 4.9 5.2 5.6 5.6	942	5.77	7.96	14.2	-10.4 -19.5 -13.6 — 8.1 -15.0	15.2 26.9 29.3 30.6 (30.3)§ 28.8		+54 +31 +15 +11 - 9	-154 -35 -32 -28 -38	-26 -46 -32 -19 -36	-128 + 11 0 - 9 - 2	

TRANSFERS OF POTASSIUM IN RENAL INSUFFICIENCY

TABLE III—Continued

TRANSFERS OF POTASSIUM IN RENAL DISEASE

TABLE III—Continued

Pt.	Diagnosis	Time	Weight	Blood NPN	Serum										Change in		Change in cellular K		
					CO ₂	Cl	Na	K	H ₂ O*	Total protein	Ca	P	by†	Extra-cellular volume†	Cell Na	Extra-cellular K	Total	With N	In excess of N
					m.eq. per liter	m.eq. per liter	m.eq. per liter	m.eq. per liter	grams per liter	grams per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	grams	liters	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.
P. DeB.	Acute glomerulonephritis	2/28	96.8	102	16.7	85.7	124.4	6.4	949	4.88	8.30	12.0	0.6	44.4	30	+32	8	+2	8
		3/1	96.8	122	14.5	84.1	121.9	7.0	947	5.06			+21.3	45.3	+30	+16	+51	+51	51
		3/2	97.5	126	14.0	82.4	124.3	7.2	944	5.47			+21.5	46.7	7	+17	+0	+18	9
		3/3	97.3	129	13.1	84.2	128.7	7.7	943	5.63			+7.5	50.5	-135	+11	+9	+22	3
		3/4	97.5	146	13.0	86.3	126.0	7.6			9.60		+9.2	47.3	-64	+24	+19	+30	14
		3/5	97.5	151	13.2	85.8	128.3	8.1					+12.6	47.3	-308	-1	+16	+22	
L. T.	Pyelonephritis	3/6												(27.0)		+62	-71	-13	58
		1/6	90.0	180	12.9	94.9	133.3	7.0	929	7.54			-5.2	28.8	-188		+48	-85	+37
		1/7	90.0	189	9.5	87.3	130.6	8.7					-36.0	(11.3)					
R. S.	Carcinomatous ureteral obstruction	11/8	45.0	245	19.4	103.4	133.0	4.9	946								+8	+7	17
		11/14				91.2	127.0	8.1	946	5.21	8.90	6.9	-4.3	(28.0)			+13	+31	+21
J. P.	Nephrosclerosis	4/14	79.1	206	13.0	98.6	133.8	7.2	946				+4.1	29.5			+20	-18	4
		4/15	78.4	218	11.9	95.2	125.0	7.1						31.0					
		4/16	78.9	239	11.0	95.9	134.3	7.2					-9.5	32.4					
		9 a.m. 4/16 4 p.m.		255		91.9		7.5											
F. S.	Nephrosclerosis Hypertensive encephalopathy	3/20		67	35.1	78.2	132.0	2.8			6.35			14.1		+102	2	-14	17
		3/21		68	32.2	77.8	122.2	2.7					+1.4	14.2		-	-1	+3	18
		3/22		65	27.9	86.0	131.9	2.6	938			9.70	+4.4	14.4		-40	+19	+11	50
		3/23		77	25.8	92.2	132.2	3.7				4.9	-	(15.0)		-		+38	12

* Based on basis of concentration of serum protein.

† Measured extracellular volume designated by parenthesis.

* Determined directly or calculated on basis of concentration of serum protein.
† The balance of nitrogen is corrected for change in NPN throughout an assumed volume of body water.
‡ Calculated forward and backward by changes in chloride balance after an assumed or measured extracellular volume designated by parenthesis.
§ Volume of distribution of mannitol.
|| Estimated weight.
Balance data are expressed per individual period rather than cumulatively.
Time as designated by date indicates end of period at which time serum analyses were made and balances for the period determined.

TABLE IV
Transfers of potassium in patients with renal insufficiency

Patient	Period	Final serum K		Average daily								
		Conc.	Time before death	Carbohydrate intake	K intake	K output	Urine vol.	Change in extracellular		Change in intracellular K		
								Vol.	K	Total	With N	Excess
	days	m.eq. per liter	hours	grams	m.eq.	m.eq.	cc.	liters	m.eq.	m.eq.	m.eq.	m.eq.
P. McG.	$\frac{1}{2}$	8.6	6	90	30	12	330	1.6*	+64	-46	-28	-18
A. M.	4	5.7	20	175	2	4	115	3.3	+14	-16	+8	-24
C. K.	1	5.8	<24	180	2	25	110	0.9	-4	-19	-12	-7
W. M.†	6	6.4	>24	146	0	9	561	-0.2	0	-9	-6	-3
W. S.	8	6.0	<24	119†	4	27	629	1.7*	+13	-36	-20	-16
P. DeB.	7	8.1	0	119†	22	3	13	0.4*	+14	+5	+25	-20
L. T.	1	8.7	0	75	2	11	95	1.8	+62	-71	-13	-58
R. S.	6	8.1	3	0	0	0	0	0.3	+8	-8	-14	+6
J. P.	2.3	7.5	15	236†	30	4	109	1.8	+18	+9	-9	+18

* Calculated by CI balance from mannitol space.

† Recovered.

‡ Insulin given.

sium was restricted and the oliguria was not extreme.

The rate of glomerular filtration of potassium was reduced far out of proportion to its rate of excretion in two of the three patients in whom the clearance of mannitol was measured. These data, which are presented in Table V with similar data from the control subjects with normal kidneys, permit a distinction between disturbances of glomerular and of tubular function. In these two patients the rate of excretion approached, if it did not exceed, the rate of filtration. The error is so great that this cannot be interpreted as definite evidence of tubular secretion. Nevertheless, the ratio of potassium excreted to that filtered certainly exceeded any such ratios in the control subjects. This was so to a lesser degree with respect to water. Defective glomerular filtration appeared to be the principal factor limiting the excretion of potassium.

The exchanges of potassium were studied in one patient with renal insufficiency with a low concentration of serum potassium (F. S., Tables II and III). During the first day of study sodium was wasted in the urine; the rate of excretion of potassium was within the range found in the potassium-deficient patients with normal kidneys. Since the glomerular filtration rate was probably markedly reduced in this patient the normal excretory rate may have been the result of proportionately decreased tubular reabsorption. Subse-

quently, when potassium was administered it entered the cells and was retained.

DISCUSSION

The results of this study indicate that elevations in the concentration of serum potassium are not infrequent in patients with renal insufficiency. Given the primary difficulty of inadequate renal excretion of potassium, the presence and degree of a rise in the extracellular concentration of the ion depends upon the change in volume of the extracellular fluid, intake of potassium, extrarenal loss of potassium, and net transfer of potassium between the extracellular fluid and the cells. In patients who are anuric and who are receiving parenteral fluids, the extracellular volume may expand. Such a change does not necessarily lower the potassium concentration since it has been shown in experimental animals that expansion of the extracellular volume in itself may lead to transfer of some potassium from the cells (24). Intake of potassium is an important factor in the accumulation of extracellular potassium as the intake readily may exceed the output or rate of excretion. To a certain extent patients are protected against the ingestion of potassium by anorexia, nausea, and pylorospasm (15). But the administration of potassium by any route may jeopardize life, and is clearly contraindicated in any patient with anuria or oliguria.

Extra-renal losses of potassium in gastrointestinal fluid may modify to a limited extent the

extracellular concentration of the ion. But this concentration is probably determined in the main by exchanges of potassium between the extracellular fluid and the cells. In agreement with other observers (25, 26) release of potassium from cells as an agonal or terminal phenomenon due to anoxia, could not be the explanation of the elevated concentration of serum potassium in most of our cases because the rate of rise was not accelerated. On the other hand, potassium was taken up and retained in the cellular phase in only a few of our cases with high extracellular concentrations. This is surprising, since in normal dogs large amounts of potassium can enter cells before being excreted (27), and since others have observed anuric patients to maintain a concentration of serum potassium within the normal range over many days (28). However, these transfers are closely related to the processes of cellular metabolism and in these patients not all of the metabolic variables were known. This fact makes it difficult to predict in a given case the net result of exchanges of potassium between the two fluid phases. The deficit of potassium present in the patients with low serum potassium and renal insufficiency probably resulted from losses of the ion in urine and in gastrointestinal fluid analogous to those observed in potassium depletion associated with normal renal function (22).

There was diminution in the concentration and excretion of potassium by the kidney in the present group of patients. This finding is in agreement with Winkler, Hoff, and Smith (15) and with Keith and Osterberg (29), who found that the clearance of potassium was low in such patients even when potassium was administered.

The rate of glomerular filtration was greatly depressed. In the organism with normal kidneys tubular reabsorption of potassium appears to be, within limits, independent of that of water. During water deprivation the concentration of potassium in urine of the dog may rise to 500 m.eq. per liter, representing a U/P ratio of 80 (30). Increased concentration ratios of lesser magnitude may be inferred from similar experiments on man (31). During water diuresis the excretion rate of potassium may be unaltered and the U/P ratio falls to 1.0 (32). To our knowledge, no U/P ratio of less than 1.0 has been reported (22). When the concentration ratio of potassium exceeds 1.0, it implies that the concentration of potassium in the reabsorbate is less than that of serum. If the concentration ratio of potassium were less than 1.0, the concentration of potassium in the reabsorbate would be greater than that of serum. Tubular secretion of potassium appears to have been demonstrated by Berliner (33) and by Gilman and Mudge (34) under certain experimental circum-

TABLE V
Factors in the renal excretion of potassium

Clinical group	Subject	Urine volume $V = H_2O_u$	Mannitol clearance $= H_2O_f$	Potassium					$\frac{K_u}{K_f}$	$\frac{H_2O_u}{H_2O_f}$
				Intake	Aver. serum conc. P	$\frac{U}{P}$	Excretion rate $UV = K_u$	Filtration rate K_f		
Normal	R. E.	ml. per day	ml. per min.	m.eq. per day	m.eq. per liter		m.eq. per day	m.eq. per day		
		1710 1860	90* 90*	250 2	4.3 3.7	29.7 4.9	218 34	558 480	0.39 0.07	0.01 0.01
Potassium depleted, normal kidneys	E. R.	3420 2030	111* 111	0 88	2.5 2.45	6.9 3.0	59 15	400 392	0.15 0.04	0.02 0.01
	R. W.	1385 830	— —	2 109	3.85 4.0	1.0 3.0	55 10	— —	— —	— —
Renal insufficiency	W. S.	986	3.1	0	5.6	8.5	47	25	1.88	0.22
	P. McG.	110†	0.7	0†	7.5	6.8	5.6†	5.2†	1.08	0.16
	A. M.	100	2.8	1	5.5	4.4	2.4	22	0.11	0.02

* Assumed to equal earlier or later determination.

† Per 16.5 hours.

stances. The present data are inconclusive with respect to evidence of tubular secretion.

As predicted by Winkler *et al.* (15), elevation of the concentration of serum potassium was always preceded by oliguria or anuria in our patients. Instances of elevation of serum potassium to toxic levels have been reported in patients who were not oliguric (17), but these patients had received exogenous potassium. In two of the three patients in whom the rate of glomerular filtration was measured, the water excreted was an abnormally large portion of the water filtered, and the potassium excreted approached if it did not exceed the potassium filtered. The restricted excretion of potassium appeared to depend on the reduction of the quantity of potassium filtered. The increase in the ratio of potassium excreted to that filtered proportionately increased the loss of potassium in the urine.

Since depression of the rate of glomerular filtration appears to be the principal limiting factor with respect to potassium in renal insufficiency, the diminished tolerance for administered potassium by such patients is readily explained. The glomerular filtration rate in advanced renal insufficiency may be 10 cc. or less per minute (35); given a normal range of concentration of serum potassium, no more than 50 to 80 m.eq. are filtered per day. Excluding cellular uptake or extra-renal loss of administered potassium, the daily increment of extracellular potassium provided by a normal diet may exceed the amount of potassium filtered. If no potassium is ingested the increment of extracellular potassium due to release of the ion from the cellular phase may not greatly exceed that which can be filtered and excreted.

Aside from restriction of intake of potassium, the treatment of hyperkalemia in the oliguric or anuric patient logically includes measures to counteract its physiological effect, to promote the transfer of potassium into cells, and to produce extra-renal excretion of potassium. The administration of calcium has been shown to antagonize the toxic action of potassium in dogs given infusions of potassium (36). The use of calcium for this purpose in man has been followed by changes in the electrocardiogram (17, 25) but has not yet been thoroughly evaluated. The administration of glucose and insulin to enhance the transfer of potassium into cells was attended with some success

in cases of hyperkalemia due to ischemic muscle necrosis (37). Apparent antagonism of the toxic action of potassium by the administration of glucose and calcium to an infant suffering from potassium intoxication has been reported (38). Glucose and insulin produced no significant effect in our patients, but was not given a sufficiently thorough and systematic trial. Infusion of hypertonic solution of sodium chloride, proposed on the theory that a low concentration of sodium in extracellular fluid leads to shift of potassium from cells, has been reported to produce electrocardiographic and clinical improvement (17). No such relationship is apparent in our data, and the use of hypertonic saline in one case was ineffectual (P. McG.). Treatment with adrenocortical substances has not been successful in animals in which treatment was begun after anuria had been produced (39). Finally, a variety of measures have been proposed to effect the extra-renal removal of extracellular potassium. Dialyses by the artificial kidney of Kolff (40), by peritoneal lavage (41), and by small intestinal lavage (42), have been shown to lower the concentration of certain constituents of blood and serum and may lower that of potassium. These are fairly elaborate procedures, but may prove to have a useful role in combatting potassium intoxication in anuric patients with potentially reversible renal lesions. Such procedures, however, may be secondary in importance to the fundamental therapy of maintenance of volume and composition of the body fluids and of the integrity of the circulation.

SUMMARY

Changes in the concentration of serum potassium were studied in 26 patients with severe renal insufficiency. The serum potassium level was abnormally high in more than one-half of the 26 patients. The greatest elevations were associated with oliguria; but oliguria was not always followed by an elevation in concentration of the ion. In 10 of the patients the intake and excretion of potassium were measured and the distribution of the ion between the cells and extracellular fluid was calculated.

The principal limiting factor in the excretion of potassium was reduction in the glomerular filtration rate.

The serum potassium was depressed in four of the cases; in the one in which it was studied the rate of renal excretion was not abnormally increased.

Potassium was taken up by the cells in only two of the nine cases with hyperkalemia; this process was not clearly accelerated by the administration of carbohydrate and insulin.

Electrocardiographic evidence of intoxication occurred before death in the four patients with serum potassium greater than 7.4 m.eq. per liter.

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COMPLEMENT AND ITS COMPONENT FRACTIONS IN CEREBROSPINAL FLUID IN INFLAMMATORY CEREBROSPINAL DISEASES

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It is generally agreed that normal spinal fluid is entirely devoid of complement, but the reports of its presence in the spinal fluid in cerebrospinal diseases are contradictory. Complement was found in the spinal fluid of all cases of meningococcus meningitis by Ward and Fothergill (1), but was reported to be absent in the same diseases by others (2-4). Ward *et al.* (1, 5) found complement to be absent in the spinal fluid of cases of influenzal meningitis. Hauptmann (6) reported that complement may be found in the spinal fluid of tuberculous meningitis, but not in meningitis of other types. An early report (7) indicated that complement was absent from spinal fluids in various pathological conditions of the central nervous system, including cases with signs of meningitis.

Present evidence indicates that complement activity results from the action of at least four components, more or less loosely combined, and present in different concentrations (8-10). When one or more of these components are missing, there is an absence of complement activity. Since the component in least concentration determines the titer of complement activity (8-10), evidence of such activity will be found when that component is present in minute amounts, and negative results will be obtained when it is entirely absent, although the other three components may be present in considerable quantities. This makes it possible to titrate each of the four components of complement independently by the addition of the other three components in excess. In accordance with the terminology adopted by Pillemer and Ecker (11) and Bier *et al.* (9) for complement and its components, whole complement as demonstrated by hemolytic activity is designated as C' and the four components are designated as C'1, C'2, C'3 and C'4. This evidence has been collected from studies of complement activity in blood serum (8-10). There are no reports of similar studies in human cerebrospinal fluid.

MATERIALS AND METHODS

Definition of reagents. A reagent used for the titration of a given component of complement does not contain the component to be titrated, but contains the three other components. Thus, reagent 1 (R1) contains C'2, C'3, and C'4, but not C'1, and is used for the titration of C'1. Reagent 2 (R2) does not contain C'2, but C'1, C'3 and C'4 and is used for the titration of C'2. Likewise R3 has all the components but C'3, and R4 all the components but C'4, being used for the titration of C'3 and C'4 respectively. Since all four components are required to produce hemolysis of sensitized red blood cells, each of the reagents by itself does not produce hemolysis.

Complement for the preparation of reagents was secured from guinea pigs, which were stunned by a blow at the base of the skull and were bled from the throat into sterile Petri dishes. The blood was left at room temperature protected from light for 30 minutes, and then placed in an icebox overnight. The serum was then drawn off, centrifuged and used immediately. No stored guinea pig serum was used in the preparation of reagents.

Preparation of reagents. With the exception of R3, the methods of preparation of reagents were in general those of Heidelberger and his coworkers, with one major exception. The preparation of zymosan, used by these workers for the inactivation of the third component, is tedious and time consuming. Powdered zymosan, which was used instead, is easy to prepare, yields an excellent reagent and lasts a long time. There were also minor variations in the preparation of the other reagents.

In the separation of complement into R1 (endpiece) and R2 (midpiece) by KH_2PO_4 or CO_2 as carried out in the laboratories of Heidelberger (9) and Ecker (10), the same preparation supplies both the R1 (supernatant fluid) and R2 (precipitate). This routine was varied since a more effective R1 was obtained by one method (KH_2PO_4) and a better R2 by the other method (CO_2).

Preparation of R1. To 9 volumes of chilled M/200 KH_2PO_4 solution 1 volume of chilled guinea pig serum was added slowly with constant mixing. After 20 minutes at 0° C. the mixture was rapidly centrifuged in an angle centrifuge at a speed of 4000 RPM. The supernatant fluid was decanted, made isotonic with 17% NaCl and neutralized with 0.1 N NaHCO_3 . Five ml. amounts of the reagent were measured out into small bottles, tightly plugged, immediately placed in the freezing compartment of the refrigerator and kept in the frozen state until used.

SOPHIE SPICER, EMANUEL APPELBAUM, AND DAVID D. RUTSTEIN

Preparation of R2. A small flask containing distilled water was placed in a beaker full of cracked ice and pure CO₂ gas was allowed to bubble through the water for 30 minutes under slight pressure. One ml. of guinea pig serum was added to 9 ml. of the CO₂ saturated water and CO₂ gas was again passed through the mixture for 20 minutes, keeping the mixture cold. The precipitate formed after 20 minutes' saturation of the serum with CO₂ was rapidly centrifuged in an angle centrifuge at a speed of 4000 RPM, the supernatant fluid was poured off and the precipitate well drained of the fluid by holding a piece of absorbent cotton to the mouth of the inverted tube. The precipitate was then redissolved in 5 ml. of

buffered saline solution. This constitutes R2 and was also stored in the frozen state.

Preparation of R3. Powdered zymen was used in the inactivation of the third component of guinea pig complement. It was prepared as follows: Approximately 5 grams of fresh yeast (Fleischmann's) were placed into a 100 ml. bottle containing 60 ml. absolute alcohol and the mixture was shaken well for 10 minutes. The suspension was then allowed to stand until the large particles settled. Only the very fine particles were collected by careful decanting. It was occasionally necessary to repeat this process to get rid of the coarser particles. The fine suspension was then centrifuged in 50 ml. tubes. The

TABLE I
Results of titration of complement components in cerebrospinal fluids from cases of meningitis compared with laboratory findings
Pneumococcus meningitis

Case No.	Date of onset of meningeal symptoms	Date spinal fluid was taken	Complement and its components*					Laboratory findings†			
			C'	C'1	C'2	C'3	C'4	Total protein in mgm. per 100 ml.	Sugar in mgm. per 100 ml.	Cell count	Micro-organisms
1	10/14	11/16	—	++++	—	—	—	38	53	M.I.	Neg.
2	12/18	12/19	—	++++	—	—	—	330	0	L.I.	Pos.
3	12/14	12/16	—	++++	—	—	—	109	25	L.I.	Pos.
4	2/4	2/6	—	++++	—	—	—	64	24	L.I.	Pos.
5	3/26	4/4	—	++++	—	—	—	86	66	L.I.	Pos.
6	—	3/26	—	++++	—	—	—	123	10	L.I.	Pos.
7	3/16	3/18	—	++++	—	—	—	225	25	L.I.	Neg.
8	3/3	4/3	—	++++	—	—	—	165	16	L.I.	Pos.
9	—	4/16	++	++++	—	—	—	246	74	L.I.	Neg.
<i>Meningococcus meningitis</i>											
10	1/6	1/11	±	++++	±	++++	++++	297	0	L.I.	Pos.
11	—	1/28	—	++++	—	—	—	83	0	L.I.	Pos.
12	1/18	2/1	—	++++	—	—	—	65	56	L.I.	Neg.
13	1/18	2/1	—	++++	—	—	—	210	57	L.I.	Neg.
14	2/24	2/26	—	++++	—	—	—	465	10	L.I.	Neg.
15	5/29	6/1	—	++++	—	—	—	82	79	L.I.	Pos.
16	6/3	6/5	—	++++	—	—	—	39	69	L.I.	Neg.
17	7/15	7/15	++	++++	—	—	—	290	12	L.I.	Pos.
<i>Tuberculous meningitis and other forms of meningitis</i>											
18	5/4	5/7	—	++++	—	—	—	58	39	M.I.	Pos.
19	5/2	5/7	—	++++	—	—	—	248	10	L.I.	Pos.
20	5/2	5/7	—	++++	—	—	—	72	29	L.I.	Pos.
21	6/18	7/10	++	++++	—	—	—	141	32	L.I.	Pos.
22†	—	3/7	—	++++	—	—	—	55	27	L.I.	Pos.
23§	4/16	4/16	—	++++	—	—	—	259	20	L.I.	Neg.
24§	4/2	5/6	—	++++	—	—	—	39	56	L.I.	Neg.
24§	5/2	5/7	—	++++	—	—	—	—	—	M.I.	Neg.

* Relative degree of hemolysis as determined by comparison with color standards
 +++++ = 100% hemolysis
 ++++ = 70% to 95% hemolysis
 +++ = 35% to 65% hemolysis
 ++ = 10% to 30% hemolysis
 + = 5% hemolysis
 — = No hemolysis

† Hem. strept. meningitis
 § Influenzal meningitis

† Normal spinal fluid
 Total protein 0-30 mgm. per 100 ml.
 Sugar 40-60 mgm. per 100 ml.
 Cell count 0-10 per cmm
 M.I. = Moderate increase
 L.I. = Large increase
 200 cells or more

alcohol was decanted and an equivalent amount of ether added. The suspension was shaken well and centrifuged at 800 RPM with the lid of the centrifuge open. The ether was then decanted and the sediment spread about the inner surface of the tube with a glass rod to facilitate drying. The zymín thus prepared was a fine impalpable white powder, weighing approximately 0.2 gram. If kept in a dry state, preferably over CaCl_2 , it will last for at least three to four months. The number of bottles to be used depends on the amount of zymín desired.

To prepare R3, one gram of powdered zymín was added to 30 ml. of buffered saline, mixed well and placed in a boiling water bath for 30 minutes, being stirred occasionally. It was then removed from the water bath, distributed equally into three 15 ml. centrifuge tubes and centrifuged for five minutes at 1500 RPM. The supernatant fluid was decanted as completely as possible. Each tube then contained the boiled zymín in a volume of about 0.5 ml. One ml. of guinea pig serum was added to each tube, stirred well with a glass rod and placed in a water bath at 37°C . for one and one-half hours. The rods were left in the tubes and used for occasional stirring of the mixture. The serum was then separated from the zymín by centrifugation. It was stored undiluted in the frozen state. Before using, the reagent was diluted 1:5 with buffered saline solution. This solution is stable for at least three to four weeks.

Preparation of R4. To inactivate the fourth component of complement, 0.12 ml. of 0.15 M hydrazine was added to 1 ml. of guinea pig serum, mixed thoroughly and incubated in a water bath at 37°C . for one hour. It was stored undiluted in the frozen state. Before use it was diluted 1:5 with buffered saline.

Titration of complement components. Whenever a sufficient amount of spinal fluid was available, the quantitative method of Wadsworth *et al.* (13) was used as in previous studies (14, 15). However, in the majority of cases only enough fluid was obtained to enable us to apply a qualitative test as follows: The presence of complete hemolysis of 0.2 ml. of a 5% suspension of sensitized sheep's red blood cells in 0.15 ml. of spinal fluid was read as 100% hemolysis. Partial hemolysis was read by comparison with color standards (13) representing percentages of hemolysis ranging from 5% to 95% in intervals of 5%.

Spinal fluid specimens. The spinal fluids used in this study were those collected by the Division of Acute Infectious Diseases of the Central Nervous System of the Bureau of Laboratories of the New York City Department of Health. These specimens were routinely examined for total protein, sugar content, cell count and the presence of micro-organisms, and the results were made available for comparison with titers of complement activity.¹

A total of 43 specimens were examined from 43 cases of cerebrospinal diseases, including cases of meningitis caused by the pneumococcus, meningococcus, *beta* hemolytic streptococcus, influenza bacillus, tubercle bacillus, as well as from cases diagnosed as poliomyelitis, measles encephalitis, postvaccinal encephalitis, mumps encephalitis, encephalomyelitis and other diseases as indicated in Tables I and III. All specimens were tested within 72 hours of collection, and the majority were tested within 24 hours of collection. All specimens were kept at refrigerator or freezing temperatures during the interval. From previous observations on blood serum (15), it is unlikely that the delay between collection and testing was a factor in the results obtained.

TABLE II

Median concentration of spinal fluid sugar and protein according to complement components present

Complements components present*	Number of cases	Median protein conc. (mg./100 ml.)	Median sugar conc. (mg./100 ml.)
Large	7	259	12
Moderate	15	82	29
Small	17	39	56

* Large = All four components present (complement activity).

Moderate = Three components present.

Small = Two or one components present.

It would have been desirable to test for complement components in every specimen of spinal fluid from each patient so as to follow the exact trend of the appearance and disappearance of individual components during the course of disease. This study was limited, however, to individual observations during the disease because of the method of collection of specimens. Nevertheless, the data as collected adequately support the conclusions reached in this paper.

In most instances, spinal punctures were performed by hospital internes and the specimens sent to the Bureau of Laboratories by messenger. The amount of spinal fluid necessary for the performance of routine examinations was removed, and the remainder forwarded for complement titration.

RESULTS AND DISCUSSION

Table I, consisting of analyses of single specimens from individual cases of meningitis, demonstrates the correlation of the changes in complement activity with those in protein and sugar concentrations. The greater the deviation of the routine laboratory findings from normal, the greater the number and the concentration of the individual components. This is further demonstrated in Table II. Thus complement titrations,

¹ Acknowledgment is made to Miss Ruth Gosling, Bacteriologist, Bureau of Laboratories, New York City Department of Health, who performed the routine examinations.

TABLE III
Results of titration of complement components in cerebrospinal fluids compared with laboratory findings from cases of non-bacterial infections

APPELBAUM, AND DAVID D. RUTSTEIN
TABLE III
Results of titration of complement components in cerebrospinal fluids compared with laboratory findings from cases of non-bacterial infections

Case No.	Date of onset of cerebral symptoms	Date spinal fluid was taken	Diagnosis	Complement components					Laboratory findings*			
				C'	C'1	C'2	C'3	C'4	Total protein in mgm. per 100 ml.	Sugar in mgm. per 100 ml.	Cell count	Micro-organisms
25	2/7	2/9	Mumps	—	++++	—	—	++++	10	59	?	Neg.
26	3/1	3/1	encephalitis	—	++++	—	—	++++	20	58	Norm.	Neg.
27	4/25	4/25	Post-measles encephalitis	—	++++	—	—	++++	93	134	Sl. I.	Neg.
28	4/30	6/8	Post-measles encephalitis	—	++++	—	—	++++	26	50	Norm.	Neg.
29	6/6	6/11	Post-measles encephalitis	—	++++	—	—	++++	40	55	Norm.	Neg.
30	6/12	6/18	Post-vaccinal encephalitis	—	++++	—	—	++++	40	66	Norm.	Neg.
31	5/26	6/3	Post-vaccinal encephalitis	—	++++	—	—	++++	13	37	Norm.	Neg.
32	—	6/20	Poliomyelitis	—	++++	±	++++	55	136	Sl. I.	Neg.	
33	5/29	6/7	Poliomyelitis	—	++++	—	++++	75	85	M.I.	Neg.	
34	7/15	7/18	Encephalomyelitis	—	++	—	++++	40	90	M.I.	Neg.	
35	6/25	7/2	Encephalitis	—	++++	—	++++	24	58	M.I.	Neg.	
36	—	7/12	Encephalitis	—	++++	—	++++	18	226	Norm.	Neg.	
37	—	4/17	Encephalitis	—	++++	—	++++	25	56	Sl. I.	Neg.	
38	6/19	6/21	Encephalitis	—	++++	—	++++	95	95	Norm.	Neg.	
39	10/8	10/10	Acute encephalitis	—	±	—	—	41	89	Norm.	Neg.	

* See legend on Table I

using this method, can be of assistance in evaluating the status of a case of bacterial meningitis. In cases of diseases of the central nervous system known not to be due to bacterial infection, complement components in cerebrospinal fluid and in lower

components were present in ways positive

* See legend on Table I

using this method, can be of assistance in evaluating the status of a case of bacterial meningitis.

In cases of diseases of the central nervous system known not to be due to bacterial infection, the complement components were fewer in number and in lower concentrations than those of bacterial infections of the meninges, as shown in Table III.

All spinal fluids available for this study were taken as a result of clinical indications. Consequently, no information is available regarding spinal fluids in individuals who are not ill. However, four fluids in which no complement components were present showed protein, sugar and cells within normal limits. This cannot be considered a control series.

In the case of bacterial infection, the manner in which the components occur in the spinal fluid is characteristic. The components of largest concentration, C'1 and C'4, most commonly occur; then the third component, and least frequently C'2, which is in smallest concentration. The greater the number of components present, the higher the titer of the individual components. When all four

components were present, the spinal fluid was always positive for whole complement. This depended on the presence of C'2, the component in least concentration, and not on the particular bacterial incitant of the disease. This same situation obtains in the case of non-bacterial infections, except that it was not possible to determine the order of C'3 and C'2, since C'3 was present in only one case and C'2 was present in none of the cases.

It is of interest that in the bacterial infections, the concentrations of the various components of the cerebrospinal fluid correspond to the relative titers of each found in human blood complement, as reported by Heidelberger and his coworkers (9). C'4 was present in greatest concentration, and then C'1, C'3 and C'2, in that order, C'2 being the one which determined overall complement titer. The results of Ecker and his associates (16) are similar except that the component in least concentration was found to be C'2 or C'3, and according to their results, either of these could be the component which determines overall complement titer. It is therefore likely from these results that the presence of complement components in the

cerebrospinal fluid, during acute inflammatory diseases, results from leakage of the components across the membrane of the choroid plexus, and not from local production of the individual components; however, proof of this must await simultaneous titrations of specimens of blood and cerebrospinal fluid.

SUMMARY

1. One or more components of complement were always found in the spinal fluid in infectious cerebrospinal disease.

2. In specimens from patients with infections of the central nervous system, an analysis of the number and concentration of the components correlates with the median values of abnormal concentrations of protein and sugar.

3. In specimens obtained from cases of inflammatory diseases of the central nervous system not due to bacteria, the number and concentration of the components are less than those occurring in the bacterial diseases.

4. In inflammatory diseases of the central nervous system, C'4 and C'1, the components present in largest concentration, occur most frequently. C'3 and C'2 follow in order of occurrence and in concentration.

5. C'2 is found in least frequency and then only in low titer. It is this component which determines total complement activity of the cerebrospinal fluid, since it is only found when the other three components are present.

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THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE IN CONGENITAL ADRENAL HYPERPLASIA WITH VIRILISM AND IN CUSHING'S SYNDROME TREATED WITH METHYL TESTOSTERONE¹

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There have been several reports of the metabolic effects of adrenocorticotrophic hormone in normal individuals (1-3) and in patients with Addison's disease (1), or panhypopituitarism (4). This trophic hormone has little or no effect upon the metabolic processes in Addison's disease. In normal individuals and in cases of panhypopituitarism there may be profound effects. The changes which occur in the first four hours after a single injection of 25 mg. have been studied by Thorn and his coworkers (1). The hematological changes consist of a shift from mononuclear to polymorphonuclear cells and a fall in the number of eosinophils. The metabolic changes are reflected in the urinary excretion. Using creatinine as a reference there is a rise in uric acid, potassium, chloride and sodium.

Browne and his group (2) have studied the effect of large doses, 210 mg., of adrenocorticotrophic hormone administered over a 24-hour period to a normal patient. They found oliguria, weight gain, increased excretion of 17-ketosteroids and neutral reducing lipids, increased excretion of potassium and retention of sodium and chloride. The retention of sodium was apparent during the 24 hours of treatment and more marked during the 24 hours immediately after treatment. There was also an increase in the fasting blood sugar from 98 to 148 mg. %, glycosuria and a slight rise in the urinary output of nitrogen.

The effect of long range treatment with adrenocorticotrophic hormone has been studied by Thorn and his group, 40 mg. a day for four to six days (1); by Mason *et al.*, 25-100 mg. a day for 12

days (3); and by Albright, Forbes and Bartter, 100 mg. a day for six days (4). Mason *et al.*, working with Li's hormone isolated from sheep glands, noted an increase in the excretion of 17-ketosteroids and neutral reducing lipids but no significant alteration in electrolyte metabolism. However, Thorn's group and Albright, Forbes and Bartter, working with adrenocorticotrophic hormone isolated from hog pituitaries, noted in all cases an increase in the excretion of potassium and decrease in the excretion of sodium and chloride during the administration of the hormone. Although the retention of sodium and chloride was more marked during the second 24 hours of treatment it also occurred consistently during the first 24 hours. Other changes were a rise in the excretion of 17-ketosteroids and neutral reducing lipids, a rise in the fasting blood sugar and an increased excretion of nitrogen. Albright, Forbes and Bartter studied also the effect of adrenocorticotrophic hormone isolated from sheep glands and observed the same effects as with the hog gland preparation.

There have been no reports on the effect of adrenocorticotrophic hormone upon the respiratory quotient and no studies upon its effect in Cushing's syndrome or adrenal hyperplasia with virilization. During the past year we have been able to administer adrenocorticotrophic hormone to one patient with Cushing's syndrome who was being treated with methyl testosterone, and to two girls with congenital adrenal hyperplasia causing pseudohermaphroditism. Studies of the respiratory quotient as well as other metabolic effects and the renal excretion of steroids and electrolytes were performed. The results were quite unusual and despite the small number of cases studied are being reported at this time.

METHODS

The patients were placed on a constant diet which approximated the normal intake as closely as possible.

¹ This work was made possible by a grant from the American Cancer Society for studies on the relationship of the pituitary hormones, thyroid hormone and the steroid hormones of the adrenal glands and gonads to normal and abnormal growth, and by a grant from the Commonwealth Fund for the study of endocrine problems in childhood.

TABLE I
Composition of the diet

Syndrome	Carbo- hydrate	Fat	Pro- tein	Calo- ries	Sod- ium	Potas- sium	Chlo- ride
	gm.	gm.	gm.	cal.	m.eq.	m.eq.	m.eq.
Cushing's syndrome	265	90	90	2,200	80	106	70
Adrenal hyper- plasia case B	220	80	60	1,800	126	85	100
Adrenal hyper- plasia case D	225	80	60	1,800	112	84	100

The protein, fat and carbohydrate composition of the diet as well as the sodium, potassium and chloride content were calculated from conventional sources. Each patient was given one gram of salt a day to use in seasoning food. This is included in the figures for the diet presented in Table I. The fluid intake was not restricted because of the variable warm weather.

Venous blood was taken for chemical and hematological studies 24 hours before the start of treatment, 48 hours later at the end of the 24-hour treatment period, and again 48 hours later. The respiratory quotient and metabolic rate were determined before breakfast and in the afternoon following a rest period of two to three hours. Urine was collected in 24-hour periods and preserved with thymol and acetic acid.

No studies were made during the first three days on the constant diet. The next three days served as a control period and treatment was given over the next 24 hours. The following 24-hour period was kept separately

to see if there was any carryover or rebound from the treatment period. The following three days served as a post-treatment control period.

The gas analyses were made with a Scholander apparatus (5) on samples of expired air collected in a Douglas bag. Ketosteroid excretion was measured by colorimetric estimation with dinitrobenzene in alkaline alcoholic solution (6). The neutral reducing lipids of the urine were measured by the phosphomolybdic acid color reaction (7) performed on extracts obtained by chloroform extraction and subjected to benzene-water partition (8) but not Girard's separation.² Other chemical determinations were performed by conventional methods (9) except for sodium and potassium which were estimated with the flame photometer (10). Ordinary technics were used in the hematological studies.

The adrenocorticotrophic hormone used in these experiments was prepared from hog pituitary glands.³ Each patient was given eight intramuscular injections of 66 mg. hormone freshly dissolved in 5 cc. of normal saline at intervals of three hours. The total dose of 528 mg. was equivalent to 83 mg. of the standard preparation as assayed by the manufacturers.⁴

CASE HISTORIES

Cushing's syndrome: The patient (H. L. H. A 35977) was a boy aged 16½ years. His health was excellent

² The neutral reducing lipids are generally reported as "11-oxysteroids."

³ Kindly furnished by Dr. John R. Mote, The Armour Co., Chicago, Illinois.

⁴ Lot No. 42-B, serial No. G-59703-A, 33 mg. equivalent to 5.2 mg. standard.

TABLE II
Clinical effects of adrenocorticotrophic hormone

Syndrome	Period	Body weight	Pulse	Blood pressure	Fluid output	Metabolic rate	Respiratory quotient
		kg.	per min.	mm./Hg	cc.	cal./day	
Cushing's syndrome	Control	58.9	51	150/100	1,575	1,640	.85
	Treatment	59.2	50	130/85	1,235	1,797	.76
	Next day	59.0	51	185/110	1,580	1,652	.89
	Control	59.2	48	150/100	1,690	1,563	.90
Adrenal hyper- plasia case B	Control	30.1	101	108/57	760	1,270	.86
	Treatment	29.8	100	120/55	1,205	1,280	.82
	Next day	29.9	100	90/50	930	1,290	.87
	Control	30.0	92	100/55	600	1,360	.84
Adrenal hyper- plasia case D	Control	29.0	93	112/62	1,040	1,474	—
	Treatment	28.6	102	120/80	790	1,416	.78
	Next day	28.8	90	110/60	855	1,476	.85
	Control	28.9	90	110/60	670	1,384	.85

TABLE III
Effect of adrenocorticotrophic hormone upon urinary excretion

Syndrome	Period	Sodium	Potassium	Chloride	Nitrogen	Neutral reducing lipids	17-keto-steroid
Cushing's syndrome	Control	<i>m.eq.</i> 142	<i>m.eq.</i> 72	<i>m.eq.</i> 147	<i>gm.</i> 13.3	<i>mg.</i> 0.33	<i>mg.</i> 10.4
	Treatment	39	120	102	13.9	1.06	24.4
	Next day	38	79	66	14.5	0.69	20.2
	Control	133	76	117	14.0	0.33	8.3
Adrenal hyperplasia case B	Control	95	61	108	9.7	0.14	27.2
	Treatment	133	63	136	13.2	0.30	41.8
	Next day	72	48	88	10.4	0.09	29.2
	Control	93	68	107	10.5	0.11	26.4
Adrenal hyperplasia case D	Control	138	74	137	10.1	0.27	32.0
	Treatment	468	153	432	8.1	0.37	55.0
	Next day	81	57	105	8.9	0.33	36.0
	Control	81	42	85	6.8	0.21	35.0

until the present illness which began at the age of 11 when he was a thin and energetic child. In the course of the next year he gained 50 lbs., developed a round "moon" ruddy face, purplish striae on the trunk, acne and excessive hair. The glucose tolerance was of a diabetic type but glycosuria did not occur. His blood pressure was 160/100. X-rays showed osteoporosis and a calculus in the right kidney. At the age of 12 a right ureterolithotomy and a left nephrolithotomy were performed. The adrenals were explored and found to be grossly normal. A biopsy showed hyperplasia of the

reticular zone of the cortex. He was given 20 doses of 95 R.U. of radiation to the pituitary. In 1945 at the age of 14 he was readmitted and found to have a urinary excretion of 2.9 mg. of neutral reducing lipids and 27-37 mg. of 17-ketosteroids. He was then placed on 30 mg. methyl testosterone daily and the neutral reducing lipids fell to 0.58 mg., while the 17-ketosteroids fell to 12 mg. Treatment with methyl testosterone was continued up until the time of the present study, throughout the experiment and after discharge from the hospital. At the time of these studies the classical picture of Cushing's

TABLE IV
Effect of adrenocorticotrophic hormone upon blood chemistry

Syndrome	Period	Sodium	Potassium	CO ₂ comb. power	Chloride	Cholesterol	Sugar
Cushing's syndrome	Control	<i>m.eq./l.</i> 137.5	<i>m.eq./l.</i> 4.7	<i>m.eq./l.</i> 28.7	<i>m.eq./l.</i> 105.0	<i>mg. %</i> 375	<i>mg. %</i> 76
	Treatment	136.6	5.0	27.0	102.3	327	118
	Control	135.3	4.9	26.6	105.6	345	80
Adrenal hyperplasia case B	Control	136.0	3.9	25.0	110.2	177	84
	Treatment	139.0	4.3	24.2	104.7	165	92
	Control	133.5	4.4	26.2	102.0	160	104
Adrenal hyperplasia case D	Control	137.5	3.8	26.6	104.0	120	88
	Treatment	135.5	4.2	25.8	102.0	121	88
	Control	141.5	3.8	27.0	102.2	125	80

TABLE V
Hematological effects of adrenocorticotrophic hormone

Syndrome	Period	Hematocrit	Leucocytes	Polymorpho-nuclears	Eosinophils	Mononuclear cells
Cushing's syndrome	Control	mm. 47.2	per cmm. 5,400	per cent 69	per cent 3	per cent 28
	Treatment	49.0	14,400	87	1	12
	Control	48.0	9,150	67	0	32
Adrenal hyperplasia case B	Control	43.5	6,400	35	0	65
	Treatment	45.7	9,800	56	5	39
	Control	45.4	5,600	28	9	63
Adrenal hyperplasia case D	Control	36.8	3,850	49	1	50
	Treatment	37.8	6,900	53	2	45
	Control	34.8	6,800	44	8	48

syndrome had not changed materially although there had been no apparent progress of the disease. The weight was 133 lbs. and the height 55.3 inches. Obesity and dull red striae were still present. The blood pressure was 150/100. The testes measured only 1.8×3 cm. X-rays showed a bone age of 15 years and slight osteoporosis. The laboratory data are given in Tables II-V.

Congenital adrenal hyperplasia with female pseudohermaphroditism. Case B. The patient (H. L. H. A 47344) was a girl aged $6\frac{1}{2}$ years. Mother, father and a younger sister are alive and well. General health excellent except for the present illness and an attack of measles one month before this hospitalization. Enlarged clitoris and absence of a vaginal orifice was noted at birth and pubic hair appeared at the age of three. At five she was found to have a height age of seven and a bone age of 11 with an I.Q. of 120. The clitoris was amputated and a urogenital sinus was demonstrated. At the time of these studies the weight was 63 lbs. and the height 56 inches. There was abundant pubic and axillary hair and the voice was deep. There was marked acne and seborrhea. The laboratory data are shown in Tables II-V.

Congenital adrenal hyperplasia with female pseudohermaphroditism. Case D. The patient (H. L. H. A 30453) was a girl aged $6\frac{1}{2}$ years. Mother and father are well and a younger sister is quite normal but two younger brothers have adrenal hyperplasia with precocious secondary sexual development but small testes. At the age of 12 days a diagnosis of pseudohermaphroditism was made. At the age of 19 months a urogenital sinus was demonstrated and a laparotomy performed. This revealed normal uterus and ovaries and normal sized adrenals. At the age of five years pubic hair appeared. At the time of these studies the weight was 59 lbs. and the height 55.5 inches. There was abundant pubic and axillary hair and the voice was unusually deep. There was no pigmentation although the skin was tan

in color. Laboratory examinations are shown in Tables II-V.

RESULTS

From Table II it may be seen that there was very slight rise in the blood pressure of the two cases of adrenal hyperplasia, and no rise in the case of the treated Cushing's syndrome until the day after treatment was completed. There was little change in the pulse rate and no febrile response. The girls with adrenal hyperplasia lost .3 to .4 kg. of weight during the 24-hour treatment period while the boy with Cushing's syndrome gained approximately the same amount, .3 kg. The fluid intake was not constant nor accurately measured. There was no consistent change in the metabolic rate of the girls with adrenal hyperplasia, while there was a slight rise in the case of Cushing's syndrome.

The rise in excretion of neutral reducing lipids, Table III, in the cases of adrenal hyperplasia was small, from .27 to .37 mg. per 24 hours in one case, and from .14 to .30 mg. per 24 hours in the other, while in the case of Cushing's syndrome the rise was more marked and lasted longer. It averaged .33 during the control period and rose to 1.06 on the day of treatment, remained at .69 the next day and then averaged .33 mg. per 24 hours for the next three days. The fall in respiratory quotient in the cases of adrenal hyperplasia was also less marked than in the case of Cushing's syndrome. In the latter, the respiratory quotient dur-

ing the control period averaged .85, immediately after treatment fell to .76, rose to .89 the next day and then averaged .90 the next three days.

There was no rise in the fasting blood sugar in the cases of adrenal hyperplasia, but in the boy with Cushing's syndrome the fasting level of 76 mg. % was increased to 116 mg. % after the injection of adrenocorticotrophic hormone, and fell again the next day to 80 mg. %, Table IV. The urinary excretion of nitrogen remained relatively constant except in one of the cases of adrenal hyperplasia where there was an increase on the day of treatment.

Although the daily excretion of 17-ketosteroids before treatment was much higher in the girls with adrenal hyperplasia and virilism than in the case of Cushing's syndrome, following adrenocorticotrophic hormone there was also a greater rise in the urinary output, *i.e.*, from 27 to 42 mg. in one case, and from 32 to 55 mg. in the other. The rise in the case of Cushing's syndrome persisted for a longer time, the excretion being 10.4 mg. daily during the control period, 24.4 on the day of treatment, 20.2 the next day and then averaging 8.3 mg. for the next three days.

There was a moderate increase in the excretion of potassium on the day of treatment in the case of Cushing's syndrome and in one of the cases of adrenal hyperplasia. All three cases showed a decreased potassium excretion on the day following treatment.

The boy with Cushing's syndrome showed a very marked retention of sodium on the day of treatment, an effect which carried over to the next day. The sodium excretion during the control period averaged 142 m.eq. per day, fell to 39 m.eq. on the day of treatment, remained at 38 m.eq. the next day, and then rose to 133 m.eq. a day for the last three days of the experiment. This retention of sodium was so marked that there was a decrease in the chloride excretion despite the rise in potassium excretion.

On the other hand, both cases of adrenal hyperplasia with virilism showed an increased excretion of sodium on the day of treatment. In one of the cases the sodium excretion rose from 138 m.eq. a day to 468 m.eq. on the day of treatment and dropped to 81 m.eq. a day for the following four days. With the increased sodium excretion there was also an increase in the chloride excretion. The

girl who showed the marked sodium and chloride diuresis manifested a slight drop in serum sodium and chloride, from 137.5 to 135.5 m.eq. per l. and from 104.0 to 102.0 m.eq. per l. respectively.

Only in the case of Cushing's syndrome was there a significant change in the serum cholesterol which fell from 375 to 327 mg. %, and then rose to 345 mg. %. In this case the total serum fat also fell from 1120 to 1070 mg. %, and then rose to 1250 mg. %.

All three cases showed a slight rise in the total white blood cell count and a shift from mononuclear to polymorphonuclear cells, Table V. The boy with Cushing's syndrome manifested a fall in the percent of eosinophils at the end of the treatment period, while the two girls showed a delayed rise in the eosinophil count when it was taken during the post treatment period. The eosinophil percentage was determined by blood smears rather than by direct count so that minor changes are not significant.

DISCUSSION

The boy with treated Cushing's syndrome responded to the injections of adrenocorticotrophic hormone in much the same fashion as a normal individual (1, 2). The rise in 17-ketosteroid and neutral reducing lipid excretion was of approximately the same magnitude and duration (two days) as with normal individuals. It should be noted at this point that this patient had received pituitary radiation in the past and was given 30 mg. methyl testosterone daily for two years prior to hospitalization. This medication was continued throughout the experiment. Whether this patient was in partial remission or merely kept under control by the administration of methyl testosterone cannot be determined with certainty.

The sodium retention and gain in weight despite the potassium diuresis are similar to the phenomena which occur in normal individuals given adrenocorticotrophic hormone.

The increase in fasting blood sugar observed in this case is similar to that reported in normal individuals although in this instance glycosuria did not occur. McAlpine has pointed out that when glycosuria is produced the excretion of glucose is greater than could be accounted for by the increased excretion of nitrogen so that he suggested that the adrenocorticotrophic hormone might pro-

duce a decrease in the oxidation of glucose as well as an increase in the amount of gluconeogenesis (2). The fall in respiratory quotient observed in this case of Cushing's syndrome following the administration of adrenocorticotrophic hormone suggests that there may have been a decrease in the utilization of glucose or a relative increase in the utilization of fats.

The effects of adrenocorticotrophic hormone in the cases of congenital adrenal hyperplasia with pseudohermaphroditism differ in certain respects from those found by us in the patient with Cushing's syndrome, or those reported in normal and hypopituitary individuals. In the cases of adrenal hyperplasia of the androgenic type, this hormone caused a moderate or marked increase in the renal excretion of sodium instead of a sodium retention which is usually found. It is believed that this represents the first clearcut example of a trophic hormone producing a qualitatively different effect upon the same endocrine gland and suggests that the secretory activity of the adrenal in cases of congenital adrenal hyperplasia with virilism differs qualitatively from that of other conditions.

The explanation of the sodium loss is not entirely clear. In a preliminary discussion (11) it was suggested that in patients with congenital adrenal hyperplasia associated with Addisonian crises the adrenal glands might secrete steroids which promote sodium excretion rather than sodium retention. It is known that the compounds 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone promote the excretion rather than the retention of sodium (12). The fact that adrenocorticotrophic hormone did not cause as great a rise in the excretion of neutral reducing lipids in the cases of adrenal hyperplasia with virilism as it did in the case of Cushing's syndrome does not prove or disprove this hypothesis. The marked increase in the production of 17-ketosteroids is compatible with the fact that the clinical findings in these cases are principally due to excessive androgen.

SUMMARY

Adrenocorticotrophic hormone, equivalent to 83 mg. of the standard preparation, was administered over a 24-hour period to one case of Cushing's syndrome being treated with methyl testosterone

and two cases of congenital adrenal hyperplasia with virilism.

The Cushing's syndrome showed a rise in the renal excretion of 17-ketosteroids and neutral reducing lipids, a rise in the fasting blood sugar, a fall in the respiratory quotient, a polymorphonuclear leukocytosis, an increase in the excretion of potassium and a marked reduction in the excretion of sodium accompanied by a slight temporary gain in weight.

The two cases of congenital adrenal hyperplasia with virilism showed a marked rise in the renal excretion of 17-ketosteroids and only a slight rise in the renal excretion of neutral reducing lipids. There was no rise in fasting blood sugar, slight fall in the respiratory quotient, and a polymorphonuclear leukocytosis. There was a moderate to marked increase rather than decrease in sodium excretion with a slight temporary loss rather than gain in weight.

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A BRIEF SKETCH OF THE HISTORY OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

ASSEMBLED FROM ITS RECORDS BY

J. HAROLD AUSTIN, M.D.

(The William Pepper Laboratory of Clinical Medicine of the University of Pennsylvania)

The American Society for Clinical Investigation as it is now known was organized in 1908 under the name: The American Society for the Advancement of Clinical Investigation. The present name was adopted in 1916.

The story of its organization has been preserved for us by Dr. Henry A. Christian in the opening pages of the first Minute Book.

FROM THE FIRST MINUTES OF THE SOCIETY

"At the time of the meeting of the American Medical Association at Atlantic City in June, 1907, several men met in an informal conference to discuss the advisability of the organization of a society whose prime purpose should be the encouragement of medical research in this country by men engaged actively in the practice of medicine.

"As a result of this meeting a committee was organized which met at the University Club in Boston on June 17, 1907, with Dr. D. L. Edsall of Philadelphia and Drs. Wilder Tileston, J. H. Pratt, and H. A. Christian of Boston present. The general scope of the society was discussed at this time and it was decided to include in the committee Dr. W. T. Longcope of Philadelphia, Dr. Rufus Cole of Baltimore and Dr. S. J. Meltzer of New York, all of whom had been present at some of the informal discussions at Atlantic City. It was decided that a meeting should be held in New York City before the meeting of the Interurban Clinical Club for further consideration of the advisability of the organization of such a society, and before this meeting was held the committee was increased by the addition of Dr. J. A. Capps of Chicago and Dr. A. W. Hewlett of San Francisco. The names of a number of men considered eligible for membership in the society were divided between the members of the committee in order that a list of their publications during the past five years might be looked up and thus form the basis of determination of their suitability to be asked to take part in the organization of the society. It was agreed that seven of the nine members of the committee must approve each man before he could be considered eligible to receive a notice explaining the aims and objects of the society and inviting him to be present at the meeting for organization.

"On November 7, 1907, a second meeting of the committee was held at the Manhattan Hotel, New York, with Drs. Meltzer, Edsall, Cole, Capps, Pratt, Longcope, Tileston and Christian present. It was decided to have

the organization meeting of the society on the day previous to the meeting of the Association of American Physicians in Washington. A committee of three to draft a constitution for presentation at the meeting of organization was appointed as follows—Dr. W. T. Longcope, Dr. S. J. Meltzer and Dr. H. A. Christian.

*New Willard Hotel, Washington, D. C.
May 11, 1908*

"At 3:30 p.m. the following gentlemen: Drs. D. L. Edsall, S. J. Meltzer, A. W. Hewlett, Wilder Tileston, E. Libman, J. A. Capps, T. C. Janeway, W. J. Calvert, J. L. Miller, J. Sailer, Martin Fischer, E. A. Locke, and Mark Richardson, met in response to a letter of invitation sent out by a committee composed of the following: D. L. Edsall, H. A. Christian, S. J. Meltzer, J. H. Pratt, W. T. Longcope, Rufus Cole, Wilder Tileston, J. A. Capps, and A. W. Hewlett, for the organization of a new society whose proposed scope had been outlined in a circular letter.

This meeting was called to order by Dr. D. L. Edsall of Philadelphia.

"Dr. Edsall was then elected temporary chairman; Dr. H. A. Christian of Boston, temporary secretary.

"The meeting proceeded to the consideration of the Constitution prepared by its committee, and with a few modifications of this adopted it as the original Constitution. Officers and Council to serve through the first Annual Meeting were elected.

"The charter members were:

Ashford, B. K., U. S. Army
Baldwin, E. R., Saranac Lake
Calvert, W. J., Columbia, Mo.
Capps, J. A., Chicago
Christian, H. A., Boston
Cole, Rufus, Baltimore
Cowie, D. M., Ann Arbor
Edsall, D. L., Philadelphia
Emerson, C. P., Baltimore
Emerson, Haven, New York
Fischer, Martin, San Francisco
Foster, N. B., New York
Hewlett, A. W., San Francisco
Howland, John, New York
Hunt, J. R., New York
Janeway, T. C., New York
Libman, E., New York
Locke, E. A., Boston
Longcope, W. T., Philadelphia
McCarthy, D. J., Philadelphia

Meltzer, S. J., New York
 Musgrave, W. E., Manila, P. I.
 Pratt, J. H., Boston
 Richardson, M. W., Boston
 Rosenow, E. C., Chicago
 Sailer, J., Philadelphia
 Simon, Charles E., Baltimore
 Steele, J. D., Philadelphia
 Strong, R. P., Manila, P. I.
 Tileston, Wilder, Boston
 Wallace, G. B., New York."

The Constitution with its eight amendments is given here, for in the printed copies only the Constitution as amended up to 1916 is to be found so that the early form tends to be lost from sight. The first printed copy in 1916 substituted the first three amendments adopted in 1911, 1913 and 1916, respectively, for the original form of these parts.

When in 1924 the next amendment was adopted it was designated as Amendment I and subsequent amendments have been successively numbered. It was probably forgotten that three prior amendments had been made.

THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION CONSTITUTION

ARTICLE I. NAME

The name of this organization shall be "The American Society for the Advancement of Clinical Investigation."

[See Amendment (1916).]

ARTICLE II. OBJECTS

The objects of this Society shall be the cultivation of clinical research by the methods of the natural sciences; the unification of science and practice of medicine; the encouragement of scientific investigation by the practitioner and the diffusion of a scientific spirit among its members.

ARTICLE III. MEMBERSHIP

Section 1. *Eligibility.*

Any practicing physician residing in the United States or Canada who has accomplished a meritorious, original investigation in the clinical or allied sciences of medicine, and who enjoys an unimpeachable moral standing in the medical profession, is eligible to membership.

Section 2. *Obligations.*

Members must manifest a continuous interest in the objects of the Society (1) by carrying out and

reporting some original investigation in medicine at least once in three years; (2) by attending at least every other annual meeting. The neglect of either of these two obligations without an explanation acceptable to the Council carries with it forfeiture of membership. (3) By being active in the diffusion of the principles of the Society, particularly among the students who come under their charge.

[See Amendment IV (1939).]

Section 3. *Nominations and elections.*

Nominations to membership signed by two members who are not officers of the Society must be made in writing at an annual meeting of the Society, and within three months after the time of nomination the nominators shall transmit to the Council letters stating the qualifications of the nominee and also a list of his professional positions and publications. The Council shall report those nominations which are approved by a two-thirds vote to the next annual meeting for election. An affirmative vote of three-fourths of the members present shall be necessary for the election of members.

[See Amendment (1913-15).]

Section 4. *Expulsion.*

Members may be expelled also for reasons not especially mentioned in the Constitution by a three-fourths vote of the total membership. The vote shall be by secret ballot.

[See Amendments I(1924), II(1929), III(1930), V(1941).]

ARTICLE IV. OFFICERS

Officers of the Society shall be a President, Vice-President, Secretary, and Treasurer, all to be elected as follows: each year a Councillor shall be elected to serve three years. However, at the first meeting three Councillors shall be elected, one of whom shall serve one year, one two years and one three years. The order of retirement of the first Councillors is to be determined by lot. A retiring officer, with the exception of the Vice-President, the Secretary and the Treasurer, shall not be eligible for any office until the expiration of one year. These officers are to be nominated by a committee of three appointed by the President, and the election shall be by secret ballot.

[See Amendment (1911).]

ARTICLE V. DUTY OF OFFICERS

The duties of President, Vice-President, Secretary, and Treasurer shall be those usually assigned to those officers. The Secretary shall also arrange for the publication of abstracts of papers presented at the meetings of the Society.

ARTICLE VI. COUNCIL

Council shall consist of the President, Vice-President, Secretary, Treasurer, and the three Councillors. Four members shall constitute a quorum. It shall be the duty of the Council to supervise the affairs of the Society, to make all the arrangements for the annual meeting, to report on the work of the members, to consider all nominations for membership and to report on them at the meeting at which they shall be balloted on.

ARTICLE VII. MEETINGS

The Society shall hold one general meeting annually.

ARTICLE VIII. QUORUM

Any number of members at the appointed time of the annual meeting shall constitute a quorum for the transaction of ordinary business. For the election of members, fifteen shall be necessary for a quorum, and for altering the Constitution forty per cent of the membership shall be necessary.

ARTICLE IX. ANNUAL DUES

The annual dues of the Society shall be such sum as the Council from time to time may fix. The non-payment of dues for three consecutive years carries with it the forfeiture of membership.

ARTICLE X. AMENDMENTS TO THE CONSTITUTION

Amendments to the Constitution must be proposed in writing by five members at an annual meeting, and must be acted upon at the succeeding annual meeting, notices of which shall contain an announcement of the proposed amendment, and such amendments shall require for their adoption an affirmative vote of three-fourths of those present.

AMENDMENTS

1911: Article IV amended to read:

ARTICLE IV. OFFICERS

Officers of the Society shall be a President, Vice-President, Secretary, and Treasurer, all to be elected annually. In addition each year a Councillor shall be elected to serve three years.

However, at the first meeting three Councillors shall be elected, one of whom shall serve one year, one two years, and one three years. The order of retirement of the first Councillors is to be determined by lot. All officers are ineligible for re-election except the Secretary and Treasurer, who may be re-elected twice, the total period of continuous service not to exceed three years. These officers are to be nominated by a committee of three appointed by the President, and the election shall be by secret ballot.

1913 (with confirmation 1915): Article III, Section 3, to read:

Section 3. *Nominations and election.*

Nominations to membership may be made in writing to the Secretary at any time. Nominations must be signed by two members, who are not officers of the Society, and must be accompanied by a statement of the qualifications of the nominee and a list of his professional positions and publications. All names thus placed in nomination prior to January 1st preceding each annual meeting shall be considered by the Council, which shall pass upon the qualifications of the candidates and report to the Society for election at the annual meeting such names as it shall approve by a two-thirds vote. An affirmative vote of at least three-fourths of the members present shall be necessary for election.

The Council shall also report to the Society a list of candidates whose names shall be continued in nomination.

1916: Article I amended to read:

ARTICLE I. NAME

The name of the Society shall be "The American Society for Clinical Investigation."

1924: Amendment "I": add to Article III:

That the number of active members be limited to 200. Active members whose membership has extended throughout twenty years, will automatically be transferred to emeritus membership, and those whose membership has extended throughout ten years or more may at their own request be

transferred by the Council to emeritus membership.

[See Amendments III (1930) and V (1941).]

1929: Amendment "II": add to Article III:

In addition to the classes of membership already provided, there shall be a class of associate memberships each open upon recommendation of the Council to universities, to clinics or to other organized groups and each to continue from year to year by the approval of the Council. The dues of such memberships are to be such sums as the Council from time to time may fix and these dues shall be used for the support of the Journal of Clinical Investigation. Such memberships shall be without voting privilege.

1930: Amendment "III": amending Amendment I (1924) adding:

Active members who have reached the age of forty-five years prior to the annual spring meeting of the Society, shall automatically become emeritus members.

1939: Amendment "IV": amending Article III, Section 2, to read:

Section 2. *Obligations.*

Members must manifest a continuous interest in the objects of the Society by the following: (1) They must attend at least every other annual meeting. The neglect of this obligation without an explanation acceptable to the Council carries with it forfeiture of membership. (2) They must be active in the diffusion of the principles of the Society, particularly among the students under their charge.

1941: Amendment "V": amending Amendment I (1924):

(a) Active members, whose membership has extended throughout twenty years, will automatically be transferred to emeritus membership, and those whose membership has extended through ten or more years may at their own request be transferred by the Council to emeritus membership. [See also Amendment III (1930).]

Emeritus members shall be entitled to attend all meetings, but not to vote or hold office. They shall be exempt from dues.

(b) The Council shall have authority to recommend each year to the Society the names of properly qualified candidates equal to the number of retiring active members, or up to the number of twenty-five, depending on which number is the larger.

At the first Annual Meeting in Washington in 1909 there were 15 of the 31 charter members present. On the program were listed 16 papers including the President's address: of these 13 are recorded as having been read.

At this meeting the first President, Dr. S. J. Meltzer, outlined in his opening address the purposes for which the Society was formed. No better statement can be made than this address provides. It can be read in full in the Journal of the American Medical Association. Excerpts from it are presented here:

EXCERPTS FROM THE PRESIDENTIAL ADDRESS AT THE
FIRST ANNUAL MEETING OF THE AMERICAN
SOCIETY FOR THE ADVANCEMENT OF CLINICAL INVESTIGATION, MAY 10, 1909,
WASHINGTON, D. C.

(From J. A. M. A., 1909, 53, 508-512)

"The honor of the presidency of a society goes with the privilege of discussing some of the aims of that society. Such a privilege becomes a duty on the occasion of its first birthday. We have to show the specificity of our objects to justify the addition of a new medical society to the many already in existence. As your first president and as one of the instigators of the movement which led to the formation of this association permit me to discuss some of the problems to the solution of which this association wishes and hopes to contribute.

CLINICAL MEDICINE AS A SCIENCE

"In the first place, I wish to discuss the problem of clinical medicine as a science. In years gone by, medicine was a unit and its leaders tried to master all its aspects. With the development of scientific methods and the growth of knowledge a process of differentiation took place. Heavy branches grew out of the stem of medicine, broke off and obtained an independent existence. Anatomy with all its dependencies broke away early, then followed physiology, pathologic anatomy, pharmacology and physiologic chemistry. Bacteriology tore off the branches of etiology and established itself as an independent growth. All these offsprings of medicine are

now well established as pure sciences, they still closely affiliate with the mother-stem and are often designated as the sciences of medicine. What is left of the old stem is clinical medicine. What is the character of this residuum? It is generally designated as the practice of medicine. I am not aware that any one has had the courage to call it a pure science. Those who do not like to call it an art say that it is an applied science. According to this view, the relation of clinical medicine to the sciences of medicine is that of technology to science in general. I am afraid that this conception of clinical medicine is widespread among those who are in the practice as well as among those who are in the sciences of medicine.

"However this may be, I feel sure that such a view is logically erroneous and practically harmful. I wish to plead for an extension of the process of differentiation also to clinical medicine. I am of the opinion that clinical medicine as it exists now is made up of two constituents: one part has all the elements of a pure science and ought to be coordinate to the other pure sciences of medicine, and the other part is the real practice of medicine, an applied science which has many elements of an art. At present both parts are so closely interwoven that they present the appearance of a natural unit the splitting of which into two parts might appear to some as an impossible and undesirable process."

* * * *

"To clinical medicine is left the study of the phenomena and their sequence as they occur in a living body during the entire course of a disease. It has to interpret these phenomena by known physiologic laws, to identify them with similar phenomena experimentally produced in animals and it has to correlate the phenomena observed in the course of a certain disease during life with the anatomic changes found at the postmortem examinations in case the disease has a fatal termination. It is also one of the tasks of clinical medicine to study the influence on the onset and course of phenomena of diseases which certain means and remedies may exert whose physiologic actions are well established. In other words, the domain of clinical research comprises the study of the natural history of diseases, their physiology and their pharmacology. Can there be any doubt that these studies constitute legitimate objects of a pure science?"

* * * *

"After showing that there is a definite field for a science of clinical medicine the next question is who should be the men to carry on the research in this field? What should be their qualifications? In the first place, they must have a training fitting them to carry out investigations in conformity with the requirements existing in all pure sciences. They must not only be informed and trained in the other sciences of medicine, but must have carried on various investigations in one or more of these pure sciences, so as to become familiar with careful scientific methods and imbued with a scientific spirit. They will thus learn best how to shape a problem so as to make it amenable to a solution; how to marshal the consecutive steps of an investigation so

as to bring out the reply to the question in hand. They will learn to avoid bias in the search, to apply criticism to the findings; they will learn not to trust only few facts, not to rejoice prematurely over findings and not to be disheartened by failures. They will thus acquire the habits and the tastes of the investigator, the scientist, which may then stick to them for life.

"However, after all these preparations they must select clinical research as the main field of their scientific activity. Clinical science will not thrive through chance investigations by friendly neighbors from the adjoining practical and scientific domains. Such volunteer service which for the present is keeping up the cultivation of the unacclaimed region is most certainly very welcome. But the acclamation, cultivation and maintenance of a field of pure science of clinical medicine cannot be accomplished by chance services from volunteers; for such a purpose we need the service of a standing army of regulars. The investigator in clinical science must devote the best part of his time and intellectual energies to the cultivation and elevation of this field just as the physiologist does in his domain—or at least as he ought to do."

* * * *

"Let me recapitulate. There is a necessity for a differentiation of clinical medicine into a science and a practice. The clinical science has well-defined objects and is a most important department. It must be managed by a set of men specially trained for and almost exclusively devoted to that task. Without the development of such a department of clinical science the efficiency of the practice of internal medicine will lag behind, no matter how progressive the allied sciences of medicine are and how great their efforts to be useful to medicine may be.

THE CONDITIONS AS THEY ACTUALLY EXIST

"In conjunction with the foregoing considerations let me glance at the conditions of clinical medicine as they actually exist here and abroad. I may state that neither abroad nor here do we find a separate class of investigators confining their activity solely to the domain of the science of clinical medicine. Everywhere science and practice of clinical medicine go hand in hand. We may, however, admit at the outset that there is an enormous difference in the status of medical research between abroad and here. Let us take the development and the present status of clinical medicine of the German-speaking countries as an illustration of the conditions abroad. We shall notice that there the mother-stem of medicine managed to retain a scientific aspect despite all processes of differentiation."

* * * *

"Teaching medicine and furthering its science is a very serious business which ought to be carried on by men who are ready to devote all or most of their time to it.

"Here are problems for you to solve. The progress of clinical medicine in this country has to be accelerated again. We might reach the level which we have seen

to exist abroad. But we ought to strive to get still higher; in this field lack of modesty is a stimulating virtue. Look at the allied sciences of medicine, the level which they have reached in a comparatively short time without any tradition and only through the activity of a small group of high-minded men. The progress of medicine in this country was always carried on by the few and not by the masses. Clinical medicine must reclaim some of the brainy young men who were enticed by the sciences of medicine. You represent a new generation. You have had an education and a training in modern medicine and the allied sciences. You have a training in investigation, the constitution requires it of you. The constitution which you have adopted shows your spirit. Among its objects is 'the cultivation of clinical research by the methods of the natural sciences and the diffusion of a scientific spirit among its members.' But you will accomplish more; the constitution imposes on you the obligation to be active in the diffusion of the principles of the Society particularly among the students who come under your charge. I would recall this obligation to the minds of the members who were called within this year to chairs of medicine at influential places. The constitution does not keep you down exclusively to science, but let me tell you: beware of practice. It is a bewitching graveyard in which many a brain has been buried alive with no other compensation than a gilded tombstone."

The growth of the Society was rapid. In 1914 there were more than 70 members. Of these, 41 were at the meeting and 25 papers were read.

Steps had to be taken at that meeting to limit future programs to permit completion of the program in a single day.

Ever since there have been discussions as to the relative merit of a one-day meeting with a single program, a one-day meeting with simultaneous sessions in different fields of interest, and a two-day meeting. The second alternative has been used in a few meetings; but the first has been the usually favored procedure.

In 1924, to provide for the ever increasing group of young workers in the field of clinical investigation, the constitution was amended to limit active members to 200 and to transfer to emeritus status all whose membership had extended through 20 years, and to permit voluntary transfer to emeritus status after ten years of active membership.

This interesting step, so far as I know unique among our national scientific societies, has insured the persistence of an atmosphere of youthful vigor in this organization. This step was rendered easier by the custom, dating uninterruptedly from the first meeting, of holding the meetings of this society on the day preceding the meeting of the

Association of American Physicians and at the same place.

The Journal of Clinical Investigation

The Constitution of the Society provided for publication of abstracts of its papers.

Early there arose a desire to support an organ of publication which might set a high standard in the field of clinical investigation and encourage its growth.

Realization of this hope became possible when in 1923 the Rockefeller Institute offered to subsidize through its early years a journal to be owned and operated by the Society. Dr. G. Canby Robinson was selected as Editor. Six members of the Society were elected as the first Editorial Board. The formulation of general policies and the election of Editor and Editorial Board was made the responsibility of an Editorial Committee of 15 members each to serve three years; five of its members retiring each year, with the vacancies filled by elections by the Society at its annual meeting.

The first issue of *The Journal of Clinical Investigation* appeared in October, 1924, and subsequent numbers appeared at bimonthly intervals. Early volumes vary in the period of a year covered, for those volumes were closed whenever a prescribed quota of pages had been issued. Later the *Journal's* volume became identical with the issues of a calendar year.

When the subsidy from the Rockefeller Institute ceased, financial aid was given for several years by the Rockefeller Foundation. For the next few years many of the departments of medicine of our universities accepted associate memberships in the Society as a form of subsidy for the *Journal*.

With Volume XI in 1932 the Chemical Foundation became interested in the project through the efforts of Dr. G. Canby Robinson. It began meeting all deficits in the costs of the *Journal* and continued this until recently the growth of the subscription list has placed the *Journal* on a self-supporting basis. During the recent war the Commonwealth Fund has aided the *Journal* by a grant to facilitate publication of studies arising out of the problems of the war.

The editorial staff for the first few years of the *Journal* is not recorded in the bound files of the *Journal*, for this information appeared only on the

cover pages of those issues and these pages are discarded on binding. The following tabulation has, therefore, some reason for record.

EDITORS

G. C. Robinson	<i>Editor</i>	1924-1927
J. H. Austin	<i>Editor</i>	1927-1935
R. West	<i>Associate Editor</i>	1932-1935
	<i>Editor</i>	1935-1940
J. L. Gamble	<i>Editor</i>	1940-1947
A. M. Butler	<i>Associate Editor</i>	1940-1947
C. S. Keefer	<i>Associate Editor</i>	1943-1947
E. M. Landis	<i>Associate Editor</i>	1943-1947

EDITORIAL BOARD (six to ten members)

J. H. Austin	1924-1927
A. E. Cohn	1924-1936
R. Cole	1924-1930
W. T. Longcope	1924-1936

F. W. Peabody	1924-1927
G. C. Robinson	1924-1935
R. T. Woodyatt	1924-1928
J. H. Means	1928-1931
L. H. Newburgh	1928-1930
W. S. McCann	1930-1943
C. P. Miller, Jr.	1930-1947
J. P. Peters	1931-1947
H. L. Blumgart	1933-1947
J. L. Gamble	1933-1940
T. M. Rivers	1933-1947
F. N. Wilson	1933-1940
F. G. Blake	1936-1947
R. F. Loeb	1936-1947
R. West	1940-1943

After the inauguration of the *Journal* the Proceedings of the meetings regularly appeared in abstract in that journal, beginning with the Seventeenth Annual Meeting in May, 1925.

The earlier Proceedings appeared in abstract as shown below and reprints were usually distributed to members.

PROCEEDINGS (IN ABSTRACT) OF THE
AMERICAN SOCIETY FOR THE ADVANCEMENT OF CLINICAL INVESTIGATION

Meeting No.	Date	Place held	Reprinted from
1.	May 10, 1909	Washington, D. C.	Boston M. & S. J., 162, 462-467 (April 7, 1910)
	(Note: President S. J. Meltzer's address is reprinted in full in J. A. M. A., 53, 508-512, Aug. 14, 1909)		
2.	May 2, 1910	Washington, D. C.	Boston M. & S. J., 162, 867-871 and 900-905 (1910)
3.	May 8, 1911	Atlantic City, N. J.	J. A. M. A., 57, 148-151 (1911)
4.	May 13, 1912	Atlantic City, N. J.	J. A. M. A., 59, 137-139, 218-220 (1912)
5.	May 5, 1913	Washington, D. C.	Probably privately printed (no reprints located)
6.	May 11, 1914	Atlantic City, N. J.	Privately printed, no place given
7.	May 10, 1915	Washington, D. C.	Privately printed, no place given
THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION			
8.	May 8, 1916	Washington, D. C.	Privately printed, no place given; 1916 constitution included
9.	May 1, 1917	Atlantic City, N. J.	Probably privately printed (no reprints located)
10.	May 6, 1918	Atlantic City, N. J.	Probably privately printed (no reprints located)
11.	June 14, 1919	Atlantic City, N. J.	J. A. M. A., 73, 637-639, 714-715, 785-787, 860-861 (1919)
12.	May 3, 1920	Atlantic City, N. J.	J. A. M. A., 74, 1597-1600, 1667-1669, 1737-1738, 1793-1794 (1920)
13.	May 9, 1921	Atlantic City, N. J.	Privately printed by Press of A. M. A., Chicago
	(Note: In this report, tribute is paid to Dr. Meltzer's memory by Dr. Longcope, who briefly describes how the Society came to be organized)		
14.	May 1, 1922	Washington, D. C.	J. A. M. A., 78, 1660-1662, 1751-1754, 1838-1840, 1919-1922 (1922)
15.	April 30, 1923	Atlantic City, N. J.	J. A. M. A., 80, 1641-1644, 1727-1728 (1923)
16.	May 5, 1924	Atlantic City, N. J.	J. A. M. A., 82, 1988-1990, 2077-2078 (1924)
17.	<i>et seq.</i> : J. Clin. Invest.		

The most complete library collection of these early Proceedings is, according to the latest Union Serials List, the New York Academy of Medicine Library, which at present has the reprints for the following meetings: 1, 2, 3, 4, 6, 7, 8, 11, 12, 13, 14, 15, 16. In the same library are Lists of Members 1909, 1910, 1916 and Triennial Catalogues 1923, 1926, 1932.

So in brief is the story of the founding of our Society, of its goals and accepted functions, of its constitution gradually modified to meet the needs

of the organization, and of the institution and growth of *The Journal of Clinical Investigation* which the enthusiasm of this young Society and the generosity of sympathetic foundations have made a realized possibility.

No one can compare the first presidential address with the record of the Society's achievements and not recognize the outstanding success with which our Society has served the purposes for which it was founded.

LETTER FROM THE EDITORS

CLINICAL INVESTIGATION

The Editors are frequently confronted with the question as to what is and what is not clinical investigation. It is true that the physician who continues to develop his clinical acuity and maturity of judgment by constant observation of the patient is employing the most ancient and still a most valuable technique of clinical investigation. A natural outgrowth of the descriptive stage of medical knowledge occurred when clinical investigation became related to morphology, with anatomy and pathology providing the foreground and background between which the patient, as an example of altered structure and function, found a somewhat uncomfortable place. With rapid advances in chemistry and physics the role of disordered function was explored by those whose main contribution was providing physical juxtaposition of patient and experimental laboratory. The paraphernalia of the laboratory with rapid multiplication of apparatus and test led to the notion that the more numerous the exact facts the better was the understanding of disease. This is true. It was forgotten, however, that abstract disease, without focus in a sick person, has no meaning.

For a time clinical investigation was carried along in the expanding domain of the laboratory and to a degree was synonymous with chemical investigation. A new test was devised and there appeared in medical journals an outpouring of reports on its application to a great miscellany of conditions. Blood levels and contents, clearance tests, tolerance tests, pH, and milliequivalent became the *sine qua non*. While this extension of the physical examination into the laboratory yielded a rich harvest, a tendency arose for the laboratory datum to displace other aspects of the patient from the student's attention. There were indications that study of the part might obscure the whole. The use of instruments sometimes became an object in itself and there arose the cult of the sphygmomanometer, the electrocardiogram, the electroencephalogram, the metabolism machine, and others, each eminently useful as a stepping stone to fuller knowledge of a sick person but reaching an early state of diminished returns as minutiae were explored.

This is not to decry the advances in knowledge as such, but the fact that the clinical investigator was so often led away from the patient because of his preoccupation with a test or an implement. This state of affairs comprises specialization and has brought benefits no one would willingly discard. The danger is that in concentrating on one aspect of disease so intensively the student risks a gain in knowledge at the sacrifice of wisdom. And the wisdom which clinical investigation should supply is to look at the sick person not as the inevitable but uninteresting vehicle of a fascinating lesion or syndrome but *whole*—related to external as well as internal environment. New trends in this direction are found on many sides and the recent emphasis on emotional, social and economic factors in human sickness is a healthy sign of a return to the patient as a person.

Psychosomatic medicine, a cumbersome though useful term, epitomizes this tendency. Clinical investigation must now include this broader approach in order to obtain and correlate facts from the environment as they relate to a patient and his disease. It should be remembered, however, that investigation into such problems not only is most difficult but requires maturity and a sound working knowledge of the phenomena of health and illness. Unless the method of comparison and control which has been developed in the traditional form of clinical investigation is applied in this newer area the fragmentation inherent in a new specialty may disintegrate rather than integrate our approach to disease.

Essentially, clinical investigation is the study of the sick person, his past experiences and adaptations, and their relation to his present plight as it is manifest by deviations in structure, function and behavior, and the internal and external processes upon which they depend. Orientation exclusively to either the internal or the external events of disease, or to only portions of these two major fields for clinical investigation must lead inevitably to the dissipation of our efforts to gain that true understanding upon which must be based present treatment and future prevention of the disorders which vex both the patient and his physician.

THE EDITORS

SOME OBSERVATIONS ON THE DEVELOPMENT OF HYPOKALIEMIA DURING THERAPY OF DIABETIC ACIDOSIS IN JUVENILE AND YOUNG ADULT SUBJECTS

By L. GREENMAN, F. M. MATEER, R. C. GOW, J. H. PETERS, AND T. S. DANOWSKI

(From the Department of Research Medicine, the Children's and Presbyterian Hospitals, and the Renszhausen Foundation, University of Pittsburgh School of Medicine, Pittsburgh)

(Received for publication October 18, 1948)

It is known that hypopotassemia may appear during therapy of diabetic acidosis or coma, at times associated with muscular paralysis (1-9). This is not, however, an invariable occurrence, nor have the origins of such decreases in the serum and interstitial fluid potassium concentrations been clearly defined. It seems probable that one or more mechanisms are responsible. These include the possibilities that hypokaliemia¹ develops as a result of a continued loss of potassium in urine, that it is a manifestation of dilution of body fluids by potassium-free solutions administered during therapy, and, finally, that it results from movements of potassium into body cells. The studies herein reported evaluate the role of each of these factors in a limited number of patients, chiefly pre-adult, in whom intensive study using the balance method was possible.

MATERIALS AND METHODS

Nine patients have been studied during a total of 11 admissions for the treatment of diabetic acidosis. Two of the subjects were young adults; the remainder were children seven to 16 years of age. Over-breathing was definitely present in each case; the serum carbon dioxide content ranged from 2.6 to 10.4 m.eq. per liter on admission. All patients were conscious, though difficult to arouse. Definite vascular collapse was present in only one patient, R. J., requiring three transfusions. Anuria did not develop in any of the subjects. Insulin was administered subcutaneously to all patients, although J. K. and T. W. also received large intravenous injections initially. Data on the retention of potassium salts administered in large amounts during convalescence to eight of the cases have been presented in a previous communication (9). The studies herein reported are based on observations from the time of admission and continued until convalescence was well established. During these inter-

vals the patients received insulin, glucose, saline, and only little, if any, potassium.

The experimental procedure employed and the methods of calculation have been previously described in detail (9-12). Briefly they may be summarized as follows: the chloride space, corrected for the external balance of this ion and for changes in the chloride concentration in serum, has been used as an index of alterations in the volume of extracellular fluid. The intake and the urinary excretion of sodium, potassium, chloride, glucose, and nitrogen were measured during intervals marked at the start and finish by determinations of the values of these various components in serum. The retention or loss of these cations and of nitrogen has been apportioned between the extracellular fluid and the cells. In the case of nitrogen, suitable corrections for changes in the concentration of the whole blood nonprotein nitrogen were made. The values for the cell balances of potassium represent, therefore, changes in excess of those which might be expected with the anabolism or catabolism of cell protein, employing the usual ratio of 1 gram nitrogen: 2.38 m.eq. of potassium.

It must be emphasized that the experimental procedure and the methods of calculation employed yield only the *over-all balance* of any particular component studied. Thus it should be clear that if the over-all loss of potassium from cells during any individual period should prove to be 40 m.eq., the possibility cannot be excluded, for example, that muscle cells actually lost 70 m.eq. but that this was reduced to 40 m.eq. by a movement of 30 m.eq. into the liver with glycogen.

RESULTS

From Table I it is evident that during treatment the serum potassium declined progressively to abnormally low levels in seven of the 11 cases. In two instances in which this did not occur, patients L. B. and M. S., hypopotassemia was already present on admission. In the former the potassium level remained low, while in the latter a rise was observed. In the two other patients, J. K. (a) and W. S., potassium decreased during treatment, but remained within normal limits.²

² All patients, save R. J., had received insulin, protamine or regular, within the 24 hours preceding admission.

¹ This term has been taken to refer solely to diminished concentrations of potassium in blood serum or plasma and the contiguous interstitial fluid, and does not necessarily indicate any decrease in the total amount of potassium in these fluids.

TABLE I

Blood and serum analyses prior to and during therapy of diabetic acidosis or coma

Patient (Age-Sex)	Time from start	Body Wgt.	Blood		Serum				
			NPN	Sugar	HCO ₃	Cl	Na	K	H ₂ O
	hours	kgm.	mgm. %	mgm. %	m.eq. per liter	m.eq. per liter	m.eq. per liter	m.eq. per liter	gms. per liter
L. B. (12M)	0	30.5	—	232	6.1	97.5	120.3	3.0	—*
	5.5	30.9	27	176	13.0	105.8	128.5	—	929
	18.3	31.3	25	70	17.2	98.9	138.9	3.0	932
	19.5	—	—	—	—	102.4	141.4	3.0	930
D. C. (a) (16F)	0	52.7	65	582	4.8	89.9	122.7	6.5	899
	5.0	—	57	327	5.2	98.3	128.9	5.1	911
	10.0	—	41	154	9.8	111.5	144.1	3.5	930
	22.8	53.4	28	171	15.1	111.5	155.9	3.0	941
D. C. (b) (16F)	0	50.0	67	786	6.8	91.4	125.3	6.7	907
	5.5	—	53	456	8.5	111.2	138.6	4.8	921
	19.2	53.9	28	163	15.4	117.1	145.6	3.3	936
	—	—	—	—	—	—	—	—	—
L. D. (15M)	0	38.7	33	1189	4.1	96.5	124.2	4.0	924
	5.0	—	25	240	5.7	110.4	145.5	2.6	934
	8.0	—	22	275	9.9	111.1	147.3	2.9	941
	15.0	—	23	73	12.0	111.4	135.7	1.8	940
	23.0	38.3	24	51	11.2	109.3	143.9	1.8	943
R. J. (14M)	0	34.9	103	1395	10.4	101.9	142.1	3.7	912
	4.0	—	100	1165	17.5	115.0	152.8	3.2	916
	6.8	—	105	895	18.1	122.7	156.6	3.2	919
	10.8	—	89	736	19.6	134.6	162.2	2.9	930
	16.8	—	79	304	19.5	145.2	165.1	2.8	936
	19.2	—	79	230	20.6	137.3	161.6	2.0	933
J. K. (a) (18F)	0	50.0	32	600	8.1	94.1	135.5	5.4	916
	20.0	53.9	21	145	19.0	97.8	140.5	5.0	934
J. K. (b) (18F)	0	50.7	29	372	2.6	103.9	116.3	3.4	910
	8.8	52.6	24	266	7.9	120.3	140.3	2.9	932
	20.3	52.8	21	71	13.9	112.9	129.9	2.9	944
W. S. (14M)	0	36.2	39	470	6.7	96.3	129.9	5.5	912
	4.5	—	35	270	7.8	100.5	139.9	5.1	931
	12.2	—	21	70	14.7	114.9	149.6	4.1	943
M. S. (15F)	0	41.5†	65	667	9.3	77.6	118.6	3.4	897
	3.8	—	—	266	13.5	93.1	128.0	3.8	927
	17.0	—	36	104	17.4	107.7	144.6	3.9	943
T. W. (26F)	0	55.0‡	—†	572	5.5	101.9	142.3	4.0	—*
	9.0	—	—	364	—	112.6	143.7	3.8	934
	21.0	—	—	284	8.9	123.9	154.3	3.1	940
	25.3	—	40	112	11.8	127.8	157.6	2.8	951
J. Ko. (7M)	0	21.3	36	429	4.1	96.8	123.3	5.2	926
	6.5	—	24	348	10.6	113.9	123.3	2.7	947
	23.0	—	—	198	14.1	107.6	135.5	2.2	939
	71.0	22.8	—	298	23.6	92.7	135.1	2.1	946

* Average value of the other cases used in calculating concentrations in extracellular water.

† NPN of 60 on admission assumed for calculation of cell N balances.

‡ Represents weight obtained by calculation, using subsequent weight corrected for intake and losses of water and solids.

Throughout these early hours of therapy each patient continued to excrete urine which contained measurable amounts of potassium (Table II). In no instance did the urinary excretion of this ion

decline to zero. These findings are in keeping with those of Atchley *et al.*, whose subjects also continued to excrete potassium during the period of treatment (13). The magnitude of this loss

in the study cited was less than in the pretreatment period, but in no instance was it entirely cancelled.

Furthermore, comparison of the external and the extracellular balances of potassium, as tabulated in Table III, indicates clearly that, with but one significant exception, the cells were losing potassium as serum levels declined. Only one patient, T. W., had received enough potassium in the intake to produce a positive balance of cell potassium. It is to be noted that these negative balances of cell potassium cannot be attributed

solely to the breakdown of protein. This is apparent from the net balances of cell N corrected for changes in the NPN, as recorded in the final section of Table III. Using the 2.38:1 cell K:N ratio it may be seen that in no instance was the negative cell N balance of sufficient magnitude to account for the entire loss of cell potassium. Neither were the negative balances of cell potassium cancelled when cell N balances became positive. *It would appear therefore from the above data that, based on the methods of calculation described earlier, potassium continued to pour out*

TABLE II
Intake and output data

Pt.	Time from start	Intake					Urine output					
		Insulin	H ₂ O	Cl	Na	CHO	Vol.	Cl	Na	K	N	Glucose
	hours	units	ml.	m.eq.	m.eq.	gms.	ml.	m.eq.	m.eq.	m.eq.	gms.	gms.
L. B.	0- 0.8	30	500	76.9	76.9	0	75	0.5	5.8	5.7	0.6	0.4
	0.8- 5.5	30	600	92.3	92.3	50	194	13.0	10.7	11.3	2.1	0.9
	5.5-18.3	20	1740	76.9	76.9	140	1010	102.9	76.1	20.6	4.2	5.9
	18.3-19.5	0	0	0	0	0	70	12.1	3.9	1.4	0.3	0
D. C. (a)	0	—	—	—	—	—	940	28.2	33.8	32.3	2.3	31.1
	0- 5.0	170	3011	544.5	544.5	22	910	30.3	29.5	24.4	2.0	22.0
	5.0-10.0	20	1000	153.8	153.8	25	680	54.7	45.2	19.8	2.2	12.8
	10.0-22.8	55	2865	321.3	321.3	80	2040	270.0	200.5	61.3	9.9	34.8
D. C. (b)	0	—	—	—	—	—	190	1.2	4.4	5.3	0.5	7.2
	0- 5.5	120	2850	400.0	400.0	13	1190	47.7	44.9	39.0	4.2	31.2
	5.5-19.2	110	4075	572.6	572.6	263	3060	25.6	119.1	85.3	1.2	98.2
L. D.	0- 5.0	200	3300	507.6	507.6	0	2280	205.8	219.8	41.0	3.9	51.6
	5.0-15.0	70	2460	174.6	174.6	138	1020†	153.8	120.2	10.5	0.9	8.2
	15.0-23.0	10	810	92.3	92.3	5	1030	206.8	133.4	13.2	2.2	7.5
R. J.*	0- 4.0	200	1750	255.8	264.8	0	390	2.7	2.6	4.3	1.4	19.5
	4.0- 6.8	100	850	117.3	126.3	0	80	0.6	0.3	1.1	0.6	3.2
	6.8-19.2	200	4150	625.0	633.5	116	1305	136.1	103.0	22.5	8.0	15.9
J. K. (a)	0-20.0	240	5240	769.2	769.2	132	3490	256.6	264.9	74.0	8.6	155.1
J. K. (b)	0- 8.8	195	4050	593.0	593.0	135	2580	145.5	162.1	66.0	5.0	55.2
	8.8-20.3	0	1500	0	0	84	800	105.0	46.0	10.9	2.9	0.3
W. S.	0	—	—	—	—	—	60	7.2	5.2	2.2	—	—
	0-12.2	154	4000	615.3	615.3	75	1100	164.6	115.9	52.7	6.1	26.4
M. S.*	0-17.0	170	6255	726.3	725.7	132	1663	78.5	38.1	54.9	10.9	24.3
T. W.*	0- 9.0	330	5000	769.1	769.1	50	295	9.1	5.3	10.3	1.1	4.3
	9.0-21.0	320	6760	799.9	799.1	200	1160	138.8	72.9	20.7	5.1	6.7
	21.0-25.3	0	1575	153.8	153.8	50	220	30.1	13.5	6.2	1.4	0.2
J. Ko.*	0- 6.5	120	2000	303.4	303.4	25	900	113.8	99.0	22.9	2.7	20.9
	6.5-23.0	100	4000	581.1	581.1	125	—	—	—	—	—	—
	23.0-71.0	65	5285	588.8	587.5	221	4750†	726.6	248.9	36.1	7.0	22.4

* R. J. received 1 m.eq. of K and 2.8 gms. of N during each of the three periods. M. S. took in 7.5 m.eq. of K and 0.55 gm. of N during the 17 hours; T. W. was given 48.7 m.eq. of K during the second period; J. Ko. took 11.5 m.eq. of K, 1.36 gms. of N in milk and orange juice in addition to 160 cc. of ginger ale and 50 cc. of grape juice during the 23.0 to 71.0 hour interval. Electrolyte content of the last two items is not known.

† Patient L. D. had emesis of 240 cc., and J. Ko. emesis of 260 cc. which were not analyzed.

TABLE III
*Balances of electrolytes, nitrogen, and carbohydrate **

Pt.	Time from start	External balance						Extracellular balance			Intracellular balance†		
		Cl	Na	K	N	CHO		Fluid	Na	K	N	Na	K
		m.eq.	m.eq.	m.eq.	gms.	gms.		liters	m.eq.	m.eq.	gms.	m.eq.	m.eq.
L. B.	hours 0- 5.5 5.5-18.3 18.3-19.5	+151 - 29 - 15	+147 - 3 - 7	-17 -21 - 2	- 2.7 - 4.3 - 0.4	+ 49 +134 0		+1.0 +0.1 -0.3	+159 + 71 - 27	+ 3 0 - 1	-2.7 -3.8 -0.3	- 12 - 74 + 20	- 14 - 12 0
D. C. (a)	0- 5.0 5.0-10.0 10.0-22.8	+510 + 96 + 49	+509 +105 +117	-25 -20 -61	- 2.0 - 2.3 - 9.9	0 + 12 + 45		+3.9 -0.4 +0.5	+561 + 91 +197	+ 8 -22 - 5	+0.6 +3.3 -5.4	- 53 + 14 - 81	- 34 - 6 - 43
D. C. (b)	0- 5.5 5.5-19.2	+347 +544	+349 +450	-39 -85	- 4.2 - 1.2	- 19 +165		+1.5 +3.8	+302 +606	- 8 - 2	+0.3 +6.9	+ 46 -156	- 32 -100
L. D.	0- 5.0 5.0-15.0 15.0-23.0	+297 + 15 -117	+281 + 47 - 45	-41 -11 -13	- 3.9 - 1.0 - 2.3	- 52 +130 - 3		+1.7 +0.1 -0.8	+369 - 67 - 57	- 4 - 6 - 2	-1.9 -0.4 -2.5	- 88 +115 + 13	- 32 - 4 - 6
R. J.	0- 4.0 4.0- 6.8 6.8-19.2	+248 +114 +479	+255 +122 +518	- 4 0 -22	+ 1.3 + 2.2 - 5.3	- 20 - 3 +100		+1.3 +0.4 +2.4	+260 + 87 +413	+ 1 + 1 - 4	+2.0 +1.0 +0.6	- 5 + 35 +106	- 10 - 4 - 19
J. K. (a)	0-20.0	+505	+494	-74	- 8.6	- 23		+4.4	+647	+19	-5.0	-153	- 81
J. K. (b)	0- 8.8 8.8-20.3	+442 -108	+425 - 49	-66 -11	- 5.0 - 2.9	+ 80 + 84		+2.4 -0.1	+507 -136	+ 3 - 1	-3.3 -1.9	- 83 + 87	- 62 - 5
W. S.	0-12.2	+443	+489	-53	- 6.2	+ 49		+2.7	+489	+ 2	-1.9	0	- 51
M. S.	0-17.0	+638	+674	-48	-10.4	+108		+3.8	+677	+18	-2.6	- 3	- 60
T. W.	0- 9.0 9.0-21.0 21.0-25.3	+755 +658 +121	+757 +723 +136	-11 +28 - 6	- 1.1 - 5.1 - 1.4	+ 46 +193 + 50		+5.4 +3.6 +0.5	+768 +697 +103	+19 0 - 4	+1.0 -3.0 +1.5	- 12 + 25 + 34	- 31 + 35 - 6
J. Ko.‡	0- 6.5 23.0-71.0	+184 -140	+198 +335	-23 -25	- 2.7 - 5.6	+ 4 +199		+1.0 -0.6	+115 - 88	- 6 - 2	-1.0 -5.6	+ 84 +423	- 15 - 10

* Balance data are expressed per individual period rather than cumulatively, and are corrected for the electrolytes and N withdrawn in serum samples.
† Changes in NPN have been taken into account in calculating the balance of cell N; the K balances represent changes in excess of the transfers associated with the anabolism or catabolism of protein.
‡ 6.5 to 23.0 hour urine specimen lost and hence calculation of balances for this period is not possible.

of cells throughout these earlier hours of therapy. It is germane to the argument of this paper to declare at this point that these continued negative balances of cell potassium, even when corrections for protein metabolism have been made, represent an observed fact. Their validity cannot be denied, but rather must be reconciled with the finding that, during these same periods of study, utilization of carbohydrate or its deposition as glycogen was taking place. This interpretation is supported by the finding that the blood sugar concentrations fell progressively while carbohydrate was administered (Table I). The balance data, Tables II and III, further indicate that administered carbohydrate was, in fact, catabolized or stored.

During these same intervals of treatment the patients received parenteral fluids in the amounts indicated in Table II. In the majority of these periods, actually 16 out of 24, this resulted in a measurable increase in the extracellular volume. These changes, based upon the balance of chloride corrected for alterations in the concentration of the chloride ion in extracellular fluid, are readily identifiable in the second section of Table III as instances in which the extracellular balance of water had risen by 0.5 liter or more. This value has been taken as a reasonable approximation of a change which definitely falls outside the range of the errors inherent in these methods of analyses and calculations (10, 11, 14). It is to be noted that in the remaining eight of the 24 periods the

extracellular space either failed to change significantly, or actually declined in volume. *These findings clearly suggest that in many, but not all, of our patients the extracellular fluid was diluted by the administration of potassium-free solutions.*

DISCUSSION

The data presented have established the following facts: (1) patients undergoing therapy for diabetic acidosis continue to excrete measurable amounts of potassium in the urine, (2) during this interval, if no potassium is administered, the *over-all* balance of cell potassium remains consistently negative, even though potassium is at times deposited with protein and the carbohydrate balances become positive, and (3) fluid therapy with potassium-free solutions during this time frequently results in an expansion of the extracellular volume.

In view of the above statements it is reasonable to suggest that in any particular patient recovering from diabetic acidosis, continued urinary loss of potassium, as long as potassium-containing urine is elaborated, will be a contributory factor in the development of hypopotassemia. Similarly, re-expansion of the body fluids, particularly of the extracellular volume, by means of solutions devoid of potassium will also tend to produce low serum and interstitial fluid concentrations of potassium. Finally, the deposition of potassium with glycogen or with protein will remove potassium from the extracellular fluid, and thereby cause hypokaliemia (10, 15). Obviously, the importance of any one of these processes in the origin of hypokaliemia will vary with its magnitude and direction. Analyses of our own group of cases, based on estimation of the *total amount* of potassium in the extracellular compartment at the start and at the end of an interval during which the potassium *concentration* had dropped, indicate that the dilution of body fluid with potassium-free fluid is at times sufficiently large to account for the entire decrease in serum potassium concentration. Less often the drop appears to be explicable by a combination of dilution of body fluids and continued urinary losses of potassium. Only rarely can the low levels be explained solely by urinary losses of the cation (Table III).

In no instance, irrespective of the roles played by dilution or by renal excretion in the production of hypokaliemia, is the movement of potassium into cells precluded. This is true even though the *net balances* of cell potassium are negative. As already indicated, nitrogen was deposited with potassium in the cells in a number of the periods. Similarly, glycogen must have been deposited with potassium in the liver during the recovery phase (15). This is apparent from the *in vivo* studies of others and is supported by the positive carbohydrate balances in our own series (16). It is clear that though these processes undoubtedly occurred, they were usually not, in our series of cases, of sufficient magnitude to cancel entirely the tendency for cells to lose potassium during the early treatment and recovery phases of diabetic acidosis. The explanation for this negative net balance of cell potassium is not available. It may well be merely a continuation, at a reduced pace, of mechanisms which result in cell potassium loss during acidosis.

SUMMARY AND CONCLUSION

A small series of juvenile and young adult diabetics undergoing therapy for acidosis has been studied by means of the balance method. It appears that during this early phase of therapy dilution of body fluids by potassium-free solutions, continued urinary loss of potassium, and migration of potassium out of the extracellular phase into cells with the deposition of protein or of glycogen, even though the *over-all balances* of cell potassium remain negative, may each contribute to the hypokaliemia which develops.

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THE EFFECT OF BILATERAL STELLATE GANGLION BLOCK ON THE CEREBRAL CIRCULATION IN NORMOTENSIVE AND HYPERTENSIVE PATIENTS¹

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Stellate ganglion block has been used in the treatment of cerebral derangements thought to be associated with angiospasm, for the purpose of relieving spasm and presumably increasing the cerebral blood flow (1-4). In view of this clinical development the question of the role of the sympathetic nervous system in the control of the cerebral circulation becomes more than an academic one. The only experimental work in man has been that of Risteen and Volpitta (3) who observed through burr holes an increase in the diameter of the pial vessels following stellate block. A number of clinical observations, ascribing dramatic results of stellate block to the release of cerebral angiospasm, have appeared since Leriche and Fontaine (1) first reported their experience with two cases of cerebral embolism. With the development of the nitrous oxide method for measuring cerebral blood flow (5-7) a quantitative technique has been made available for clinical study of this problem. Quantitative data can be obtained concerning the fundamental physiology of the cerebral circulation following block of that part of the sympathetic innervation of the cerebral vessels passing through the stellate and middle cervical ganglia. This information may permit evaluation of stellate block as a means of

therapy in cerebro-vascular derangements. The following studies were undertaken in an effort to explore these problems.

METHODS

Thirteen patients were studied; their diagnoses are given in Table I. In all patients except A.S. and J. C. there was a therapeutic or diagnostic indication for performing stellate block. With the exception of R. H., all patients were ambulatory and in full possession of their faculties. R. H. had been injured three weeks prior to the study and was aphasic and disoriented at the time of study. The patients with Parkinsonism were studied as outpatients. All the hypertensive patients came from the medical wards.

Mean arterial blood pressure (MABP) was measured from the femoral artery by a damped mercury manometer attached directly to a needle in the artery. Blood oxygen and carbon dioxide analyses were carried out by the manometric technique of Van Slyke and Neill (8). Blood pH measurements were made anaerobically at 37° C. using a glass electrode. Carbon dioxide tensions ($p\text{CO}_2$) were calculated according to the nomograms in Peters and Van Slyke (8). Cerebral blood flow (CBF) was determined by the nitrous oxide method using 21% oxygen, 64% nitrogen and 15% nitrous oxide as the inspired gas mixture (6, 7). From this value and the mean arterial blood pressure the cerebral vascular resistance was calculated (6).

The patients received no medication prior to the study. Following a control cerebral blood flow measurement bilateral block of the stellate ganglia was performed using the technique described by Pereira (9). Either 1% procaine or 1% intracaine without adrenalin was used, 20 cc. of solution being injected on either side. The criteria for an effective block were: scleral and conjunctival injection, ptosis, narrowing of the palpebral fissure, miosis and anhidrosis of the forehead. The block was usually evident within five minutes, reached its height within 15 minutes and lasted from 45 to 60 minutes. When block of the second side was judged to be complete, a second cerebral blood flow measurement was performed.

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TABLE I
Effect of bilateral stellate ganglion block on blood constituents

Patient	Age	Diagnosis	Blood CO ₂ content				Blood CO ₂ tension				Blood O ₂ content				Blood pH			
			Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular	
			C*	E†	C	E	C	E	C	E	C	E	C	E	C	E	C	E
J. C.	74	Normotensive with Cirrhosis	Vol. % 47.4	Vol. % 47.5	Vol. % 52.0	Vol. % 52.9	mm. Hg 35	mm. Hg 34	mm. Hg 37	mm. Hg 38	Vol. % 16.4	Vol. % 15.9	Vol. % 10.4	Vol. % 10.3	7.45	7.45	7.44	7.42
A. S.	42	Normotensive with Syphilis	49.7	45.8	54.2	54.0	42	34	47	40	15.7	15.9	10.0	8.0	7.39	7.47	7.34	7.42
B. C.	25	Normotensive with Headache	51.3	50.5	56.2	55.6					17.8	17.6	12.4	11.8				
B. P.	44	Hypertensive	45.1	46.5	53.2	53.5	38	38	45	46	19.3	19.5	11.0	12.0	7.42	7.42	7.37	7.38
E. L.	43	Hypertensive	50.3	48.9	54.8	55.3	39	36	44	42	16.5	16.5	10.4	9.9	7.44	7.46	7.40	7.41
M. O.	48	Hypertensive	51.0	46.9	58.1	54.7	37	33	42	38	18.0	18.0	10.6	11.1	7.48	7.49	7.44	7.47
N. S.	47	Hypertensive with C.V.A.‡	51.4	49.8	58.6	57.0	37	39	42	41	17.8	18.2	9.7	10.4	7.50	7.44	7.44	7.38
M. G.	44	Hypertensive Hemiplegia	57.1	57.4	61.0	62.0	36	40	45	44	12.4	12.3	7.4	7.2	7.51	7.48	7.44	7.45
F. P.	33	Hemiplegia	49.3	49.8	56.0	56.8					16.7	16.7	8.7	8.2				
D. T.	28	Parkinson's Disease	50.3	47.9	55.4	54.7	39	40	45	46	16.9	17.2	10.3	11.2	7.43	7.40	7.38	7.36
A. P.	40	Parkinson's Disease	48.4	49.4	54.4	54.9	34	36	49	49	15.7	15.7	9.4	10.1	7.40	7.38	7.34	7.34
L. C.	35	Parkinson's Disease	44.6	40.0	49.7	48.4	41	32	43	41	17.5	18.0	11.2	9.7	7.40	7.44	7.36	7.38
R. H.	23	Head Injury	51.5	51.8	55.8	56.0					20.2	20.3	15.0	15.0				
Mean	44		49.5	48.6	55.3	54.8	38	36	44	43	17.0	17.1	10.1	10.0	7.44	7.45	7.40	7.40

* C = Control.

† E = Post bilateral stellate block.

‡ C.V.A. = Cerebral vascular accident.

TABLE II
Effect of bilateral stellate ganglion block on cerebral circulation and metabolism

Patient	(CBF) Cerebral blood flow		(CMRO ₂) Cerebral metabolic rate		(CVR) Cerebral vascular resistance		(MABP) Mean arterial B.P.		A-VO ₂ ‡		RQ§	
	C*	E†	C	E	C	E	C	E	C	E	C	E
	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.	cc./100 g./min.	mm. Hg/cc./100 g./min.	mm. Hg/cc./100 g./min.			Vol. %	Vol. %		
J. C.	44	46	2.6	2.6	2.1	2.2	93	100	6.0	5.6	0.77	0.96
A. S.	42	38	2.4	3.0	2.0	2.0	85	77	5.7	7.8	0.79	1.05
B. C.	69	66	3.7	3.8	1.5	1.6	101	105	5.4	5.8	0.91	0.88
B. P.	43	52	3.6	3.9	3.1	3.1	134	159	8.3	7.5	0.98	0.93
E. L.	45	36	2.7	2.4	4.1	5.3	182	189	6.1	6.6	0.74	0.97
M. O.	59	42	4.4	2.8	3.2	3.9	186	163	7.4	6.9	0.96	1.13
N. S.	46	42	3.7	3.3	3.0	3.4	140	142	8.1	7.8	0.88	0.92
M. G.	59	54	3.0	2.8	2.9	3.2	170	173	5.0	5.1	0.80	0.90
F. P.	33	32	2.6	2.7	2.8	2.8	91	90	8.0	8.5	0.84	0.71
D. T.	47	54	3.1	3.2	2.2	2.0	105	109	6.6	6.0	0.78	0.97
A. P.	53	65	3.3	3.3	1.7	1.5	98	115	6.4	5.6	0.95	0.97
L. C.	60	52	3.8	4.3	1.6	1.8	92	97	6.3	8.3	0.81	1.01
R. H.	39	42	2.8	2.2	2.9	2.8	97	96	7.2	5.2	0.88	0.81
Mean	49	48	3.2	3.1	2.5	2.7	121	124	6.7	6.7	0.85	0.94
Normal	54		3.3		1.6		86		6.3		0.99	

* C = Control.

† E = Post bilateral stellate block.

‡ A-VO₂ = Cerebral arterio-venous oxygen difference.

§ RQ = Cerebral respiratory quotient.

RESULTS

The data are presented in Tables I and II. It will be seen that the patients comprise a clinically heterogeneous group. In the series as a whole neither the cerebral blood flow nor the cerebral vascular resistance was significantly changed following the stellate ganglion block. However, when the hypertensive group is considered separately, there is a significant increase in cerebral vascular resistance (3.3 to 3.8, $p < 0.05$) with no significant change in the other functions studied. The predominance of hypertensive persons explains the high mean arterial blood pressures recorded. The cerebral vascular resistance is considerably elevated for the hypertensive patients in this group as previously described (10). We feel that the cerebral vascular resistance most explicitly conveys information concerning the tonic state of the cerebral vessels. It seems unlikely that changes in intracranial pressure or blood viscosity of a magnitude sufficient to affect cerebral vascular resistance occurred during these experiments (11).

DISCUSSION

Our observation that bilateral stellate ganglion block in our patients produced no measurable change in the cerebral circulation is in contrast with the presumption that this procedure leads to dilatation of the cerebral vessels and an increase in cerebral blood flow (1-4). It does not, however, prove that the cerebral blood vessels of man are without sympathetic control, nor rule out the possibility that under abnormal conditions there may be vasospasm of sympathetic origin. To prove the former, one must be certain of having excluded all sympathetic nerve impulses to the brain. This cannot be assured even for those impulses which pass through the stellate ganglia, for the local block may have been incomplete. Complete surgical extirpation of the stellate ganglia would undoubtedly be the best approach in studying the physiology of the intrinsic nervous control of the cerebral circulation (12). Local block, though admittedly less certain of interrupting the sympathetic pathways, is the technique used currently in clinical therapy. Furthermore it is by no means certain that all the sympathetic fibers to the brain pass through the stellate gan-

glia (13, 14). As for the possibility that the situation may be different under other clinical conditions, such as acute angiospasm, only further study can give an adequate answer. None of the subjects of these experiments showed any symptoms suggesting acute cerebral angiospasm.

An apparent increase in the cerebral vascular resistance of the hypertensive patients calls for comment. We feel that this was probably the result of a relatively greater flow through the facial veins causing a greater extracerebral contamination in the presence of an unchanged cerebral blood flow. On the basis of this hypothesis one would expect an increased cerebral vascular resistance, a decreased cerebral blood flow (50 to 45) and a decreased cerebral oxygen uptake (3.5 to 3.0). Although these latter changes are not statistically significant they are in a direction compatible with the thesis. That this should occur in the hypertensive group and not in the other patients may be explained by the higher mean perfusing pressure during the block in these patients. These findings indicate that the usually small error in the nitrous oxide method due to contamination (6) may be magnified when blood flow through the skin is significantly increased.

We conclude that quantitative measurements of the cerebral blood flow in man gives no indication that bilateral stellate block causes an increase in cerebral blood flow or diminution in the tone of cerebral vessels. These results are compatible with the thesis that the sympathetic nervous system plays little role in the intrinsic control of the cerebral circulation under the conditions studied (including essential hypertension). An evaluation of the mechanism of improvement following stellate block as a therapeutic measure in man cannot be made until studies have been made in patients presumed to have acute cerebral angiospasm.

SUMMARY

1. In 13 patients quantitative studies of the cerebral blood flow and cerebral vascular resistance were made using the nitrous oxide technique before and after bilateral block of the stellate ganglia.

2. There were no significant changes in any of the functions studied before and after block.

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PEPTIDASE ACTIVITY IN HUMAN SERUM FOLLOWING BONE FRACTURE

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In a previous communication (Barber *et al.* [1]) it has been shown that there exists in human serum a cobalt-activatable enzyme which splits 1-leucylglycylglycine (LGG). The rate of hydrolysis in normal adult individuals is between 5.5 and 8.5% per hour (in the previous communication the rate of hydrolysis of LGG by serum of normal adult controls was stated to be 6.1 to 7.8% per hour. This value had to be revised on the basis of more subjects investigated since then).

This investigation was originally planned for the clinical study of tissue reactions of the ageing organism. The present communication is not immediately related to this problem but seems to have considerable pathophysiological significance: following fractures there was found to be an abnormal rise in the activity of the LGG-splitting enzyme in human serum.

Ever since the studies by Cuthbertson (2) and Browne (3) and their co-workers, numerous clinical and experimental investigations have been made on the metabolic aspects of injury and repair. There have been many attempts to explain these observations on the basis of one hypothesis but so far no single mechanism has been discovered which could account for the irregularities of protein metabolism accompanying injury and repair. Therefore, every new observation is significant in spite of the fact that its functional meaning may not be clear on the basis of our present-day concepts.

METHODS AND MATERIAL

Blood was obtained by venous puncture from nine patients who had suffered fractures of varying degree. Serum was prepared immediately by centrifugation and stored for not more than 24 hours in a refrigerator. The tri-peptide 1-leucylglycylglycine was used as substrate for the determinations which were carried out in triplicate in 2.0 cc. volumetric flasks. The reaction mixture, made up of 0.2 cc. serum per cc., 0.001 M cobalt sulphate, 0.05 M LGG, was kept near pH 7.8 with 0.01 M phosphate buffer

and made up to volume with redistilled CO₂-free water. To each tube, 0.01 ml. toluene was added as preservative. Flasks containing serum and substrate controls were prepared, and a zero-time titration was carried out on 0.2 ml. aliquots of the test solutions, according to the micro-technique of Grassmann and Heyde (4). The preparations were then incubated in a water-bath at 39° for six hours, during which time three titration readings were taken. Corrections were made for the controls and the results plotted on a graph. The rate of hydrolysis was calculated from the slope of the zero-order plot of per cent hydrolysis versus time (Barber *et al.* [1]).

In one case (OT), in addition to the peptidase activity, the white blood count, differential, and sedimentation rate were investigated.

RESULTS

The results are demonstrated in Tables I and II and in Figure 1. We see that out of nine cases of fracture the value of hydrolysis was abnormally high in eight.

TABLE I
Serum peptidase activity following fracture

Name	Sex	Age	Nature of injury	Time after injury	Hydrolysis of LGG % per hr
BE	M	40	Crushing injury to left hand Loss of tip of index finger	1 day 7 days	15 10.4
BR	M	58	Compound fracture of left fibula	4 days	13
DI	M	62	Right leg fracture	1 day	12.6
KL	F	48	Fracture lower end of radius	3 hours	6.7
LE	F	29	Fractured pelvis	1 day	14
WE	F	35	Lumbar fracture Fractured arm	10 hours 6 days	14.6 8.4

The one exception was a case of fracture of the lower end of the radius. It is noteworthy that in this case the blood was obtained sooner after the injury (three hours) than in any other. In another case (lumbar and arm fracture combined) the time interval between injury and blood examination was 10 hours; in this case the rate was 14.6% which belongs to the highest range

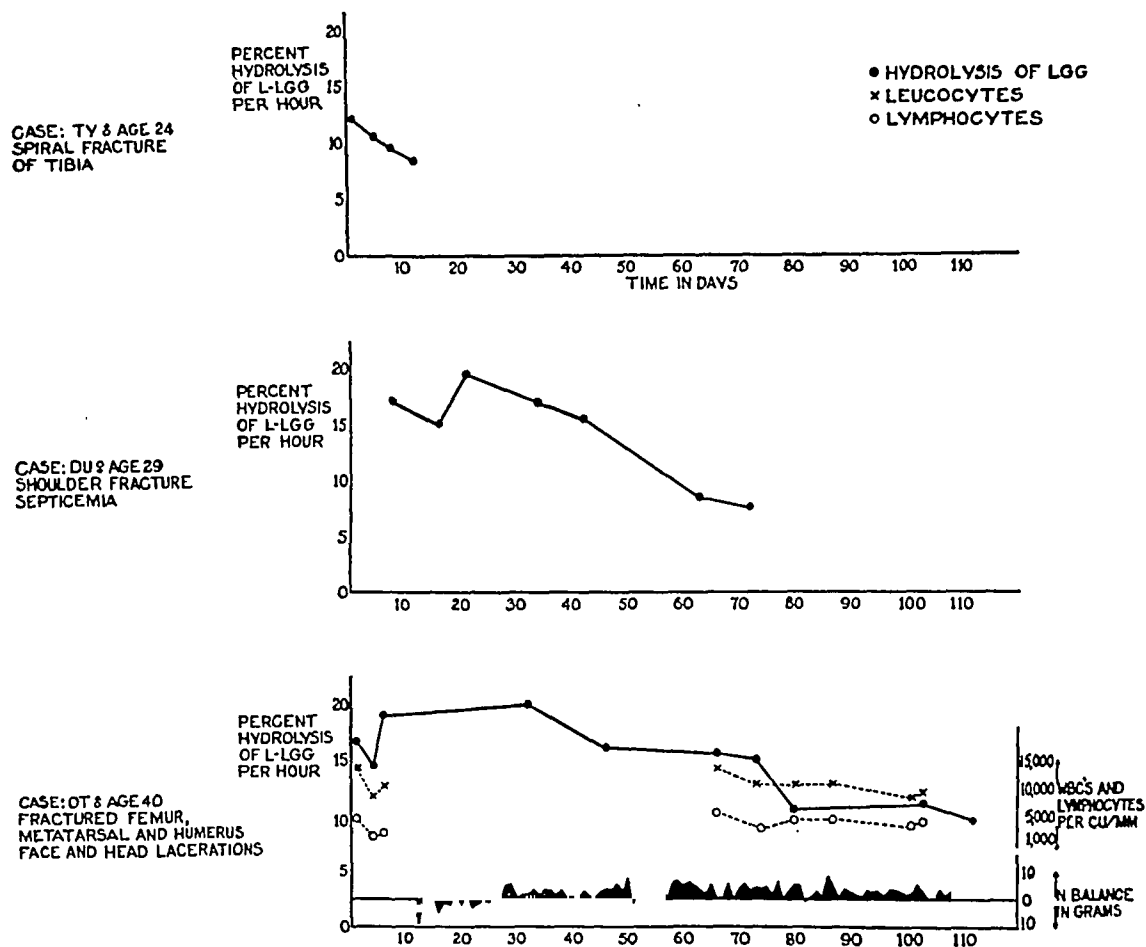


FIG. 1. GRAPHIC PRESENTATION OF THE THREE CASES WHICH WERE STUDIED LONGITUDINALLY

The solid black area in the lowest graph (Case OT) represents nitrogen balance in grams (for further explanation see text).

of the present series. In the remaining seven cases the time interval for the first blood examination ranged from 24 hours to eight days.

In the three cases in which longitudinal studies were made during the period of convalescence we see a tendency to a gradual decline from a high towards a normal value. In one case (TY) this comes close to a linear relationship between activity of the enzyme and duration of the healing process. In the other cases the return to normal is more irregular but it is noteworthy that the convalescence in these instances was complicated. In case DU septicemia occurred; in case OT there were multiple fractures with widespread lacerations. The latter case was not regarded as completely healed at the time of completion of this paper. It is known that every fracture causes an increase in the erythrocyte sedimentation rate. Hauck (5) states that a delay in the return to normal of this factor would reveal disturbances

of consolidation of the injured bone. In Table II it may be observed that in case OT the sedimentation rate is still elevated when the serum pepti-

TABLE II

*The relationship of erythrocyte sedimentation rate to serum peptidase activity in Case OT**

Day after fracture	Sed. rate	Corr. sed. rate	Compact cells	% Hydrolysis of LGG per hr.
	mm./hr.	mm./hr.	mm.	
1	31	11	33	16.2
4	68	24	25	14.5
6	69	20	25	18.8
32	—	—	—	20
47	42	36	44	16
66	40	34	45	15.5
73	40	28	40	15
80	43	28	39	10.2
103	35	43	28	11.5
110	37	32	44	9

* This patient, male, aged 40, suffered compound fractures of the left femur, left metatarsal and fracture of left humerus, with numerous head and face lacerations.

dase activity has practically reached the normal value.

DISCUSSION

We see that in eight out of nine cases of fracture the activity of the LGG peptidase of the serum was significantly increased. The one exception was a case of a relatively minor fracture in which the blood was taken sooner after the accident than in any of the remaining cases. On the other hand, the patient whose peptidase activity was highest (20% on the 32nd day) had suffered the most widespread and severe injuries. This would, at first glance, suggest a correlation between severity of injury and degree of peptidase activity; however, the number of cases is too limited to allow such a conclusion.

What is the possible mechanism of this phenomenon? Fruton (6) discussed the widespread distribution of peptidases capable of splitting LGG, in intestinal mucosa, in lymph and muscle, and in leucocytes and lymphocytes, suggesting that they may have a common origin in the leucocytes and lymphocytes invading the tissue. Holman, White and Fruton (7) observed an increase in the manganese-activatable LGG serum peptidase activity following the injection of adrenocorticotrophic hormone into mice and rabbits, and considered this to be associated with the resultant turnover of lymphoid tissue. If this is so, then our observed increases in peptidase activity during the healing of fractures might be directly correlated with the leucocytic reaction at the site of the fracture. Moritz (8) states that the reactive inflammation with exudation begins "soon after the injury has been sustained and reaches its maximum between ten days and two weeks." From experiments carried out in our laboratory, the injection of adrenocorticotrophic hormone into normal control subjects is not followed by any striking increase in the activity of the cobalt-activatable serum peptidase.¹ Moreover, in cases of acute infections there was no parallel between the numbers of circulating lymphocytes and the activity of this enzyme.

The changes in protein metabolism associated with injury and repair have been discovered comparatively late (Cuthbertson [9]). Since then a wealth of observations has been produced. From

the careful review by Beattie (10) it is quite obvious that there is no one theory on which the various metabolic phenomena associated with injury and repair could be explained. In only one of our cases (Figure 1, Case OT) was it possible to observe the nitrogen balance² together with the peptidase activity; in this case the peptidase activity began to decline with the onset of a positive nitrogen balance. Ingle *et al.* (11) demonstrated that adrenalectomized rats did not show the characteristic negative phase of the N-balance following fracture. From this it would seem that the presence of an adrenal hormone is essential for this reaction. However, as noted above, in normal human subjects we failed to obtain any striking increase in serum peptidase activity following injection of adrenocorticotrophic hormone. Thus we see that fractures are associated with a rise and return to normal of the peptidase activity of the serum during the course of healing. However, on the basis of present-day knowledge we have no definite theoretical explanation of this phenomenon. Observations like these may well lead to a procedure which would be clinically useful in the evaluation of wound healing.

SUMMARY

Eight out of nine cases of fracture showed in their blood serum an increase in the activity of the cobalt-activatable enzyme which hydrolyses LGG. In three cases studied longitudinally the rate of hydrolysis returned to normal during the course of healing. In one of these cases nitrogen balance studies were done simultaneously. In this case the activity of the enzyme began to decline when a positive nitrogen balance was established.

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² The authors are indebted to Dr. J. S. L. Browne and Dr. L. G. Johnson of the University Clinic, Royal Victoria Hospital, Montreal, for the data on the nitrogen balance study shown in Figure 1.

¹ Observations to be published.

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THE RENAL REGULATION OF ACID-BASE BALANCE IN MAN. IV. THE NATURE OF THE RENAL COMPENSATIONS IN AMMONIUM CHLORIDE ACIDOSIS¹

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The major body buffers, including bicarbonate, plasma and tissue proteins and intracellular organic phosphate complexes, can yield sufficient base within a pH range compatible with life to neutralize at most 1 mol of strong acid (1). As an immediate compensation for the invasion of the body by acid, urine of increased hydrogen ion concentration is formed, but since the pH of the urine is limited to a minimum of 4.4 to 4.7 (2, 3), the quantity of strong acid which can be excreted in free form is negligible. Acid is at first eliminated fully neutralized by base derived from the above mentioned buffers (4-6). According to Gamble the resulting depletion of base stores serves to stimulate the production and excretion of ammonia. Over a period of several days ammonia is substituted in progressively increasing amounts for fixed base in the urine, until eventually nearly all of the acid is eliminated in combination with ammonia (4-7). During recovery from acidosis the excretion of ammonia remains high, and base derived from ingested salt is retained in the body to rebuild buffer stores. Only after these stores are replenished does the excretion of ammonia decrease to normal levels.

The loss of base from both intra- and extracellular compartments during the initial phase of acidosis is accompanied by a compensatory loss of water, leading to cellular and interstitial dehydration and to reduction in circulating plasma volume (4, 6). Presumably this latter factor is responsible for the reduction in urea clearance and for the nitrogen retention often observed in severe acidosis (8). However, loss of fluid does not completely compensate for loss of alkali, for the total

base concentration of the plasma usually decreases significantly (4, 6, 9-11).

The acidosis of diabetic ketosis differs from that outlined above chiefly in the greater contribution of titratable acid excretion to total acid elimination (1, 12, 13). Because of the weakly acidic nature of beta-hydroxybutyric acid appreciable quantities of acid can be eliminated in free form in urine of maximal acidity. In severe diabetic ketosis as much as 0.15 mol of titratable acid may be excreted per day. However, providing the development of ketosis is slow, some two to three times this quantity of acid is eliminated in combination with ammonia. In rapidly developing ketosis, loss of base and dehydration may reach lethal proportions before the renal production of ammonia increases sufficiently to balance the acid load (14, 15). In chronic nephritis the renal capacity to excrete ammonia is reduced (16, 17), and since acid is eliminated for the most part in combination with fixed base, the buffer reserves of alkali suffer progressive depletion (9, 18, 19).

The present study of the response of the normal kidney to an increased load of strong acid was undertaken (a) to relate the rates of excretion of the several ions to their respective plasma concentrations, (b) to determine the nature and extent of the changes in glomerular filtration, renal plasma flow, and ionic reabsorption which underlie altered excretory functions in metabolic acidosis, and (c) to study the time relations of the renal compensations to acidosis. A number of complete though separate studies of acid-base equilibria of plasma and of urinary electrolyte balance have been made in experimental acidosis and in the acidosis of chronic nephritis and diabetes (9, 10, 12, 13, 16-18). However, in no study to date have electrolyte balances been re-

¹ Aided by grants from the United States Public Health Service and the John and Mary R. Markle Foundation.

lated to the changes in plasma composition and renal function which ultimately determine their character. The data presented below partially fill this void.

METHODS

Three types of experimental procedures were applied in this study.

(a) Two healthy adult males were maintained for periods of 15 and 16 days respectively, on a diet constant with respect to salt content and caloric value. Accurate 24-hour urine samples, preserved with toluene, were collected during these periods. Daily blood samples were drawn from a superficial vein after soaking the forearm in water at 47° C for 10 min. to render the composition of the venous blood essentially that of arterial blood. The plasma and urine samples of both subjects were analyzed for sodium, chloride, bicarbonate, pH, and phosphate. Hematocrit, urinary specific gravity and urinary content of titratable acid and ammonia were determined daily. The plasma and urine samples of the second subject were in addition analyzed for potassium, calcium, sulphate, and protein, while the urine was further tested for magnesium, organic acid, creatinine, creatine, and amino nitrogen. In

the first experiment sodium was analyzed by the method of Butler and Tuthill (20). In the second experiment, sodium and potassium were determined by an internal standard flame photometer² with an accuracy within ± 2 per cent, as checked against the method of Butler and Tuthill. Magnesium was precipitated as the ammonium phosphate and the latter ion determined colorimetrically (1). Plasma proteins and specific gravity were determined by the copper sulphate method of Phillips *et al.* (21). Organic acid in urine was determined by electrometric titration of aliquots between limits of pH 8.0 and 2.7 following removal of phosphate with calcium hydroxide. Titrations were corrected for creatinine content of the urine (1). Other methods employed have been described in previous communications (3, 22). The first five days of each experiment constituted the control period; the second five days, the period of acidosis; and the remaining five or six days, the recovery period. The first subject ingested 15 gms. of ammonium chloride daily in divided doses for the initial two days of acidosis followed by 10 gms. daily for the

² We are indebted to Dr. Robert L. Bowman of the Department of Medicine of New York University College of Medicine for the design of our flame photometer and for his helpful advice during its construction.

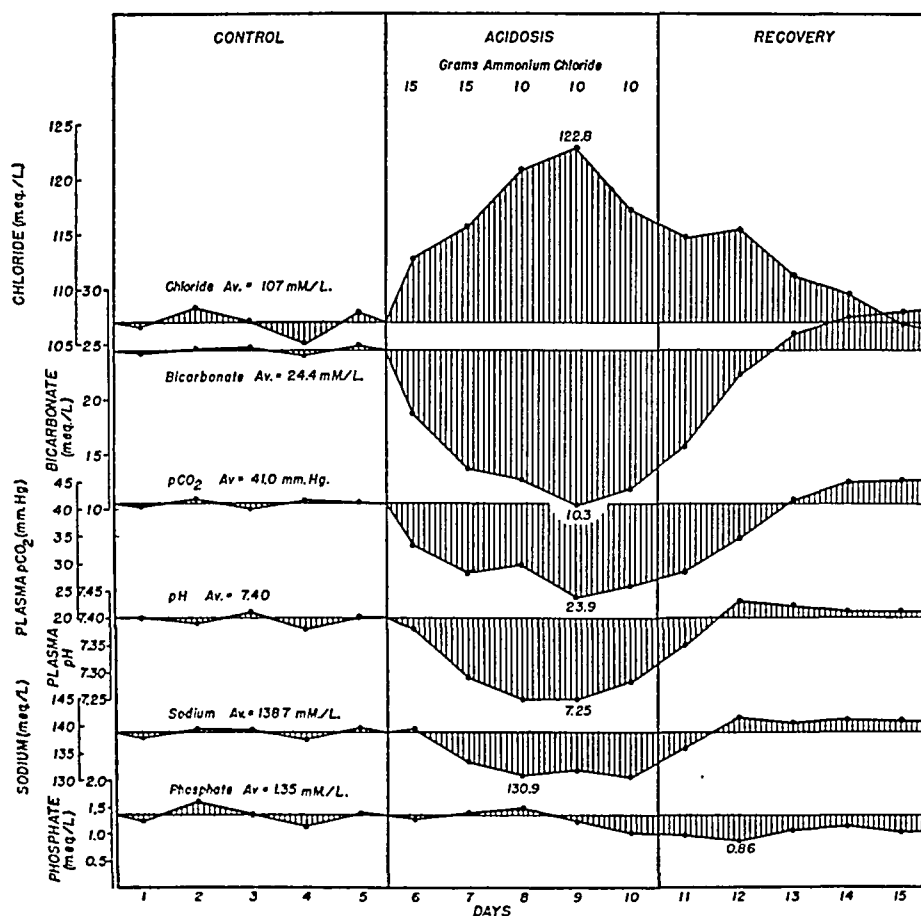


FIG. 1. CHANGES IN THE IONIC COMPOSITION OF THE PLASMA OF SUBJECT P WHICH RESULTED FROM THE INGESTION OF 60 GMS. OF AMMONIUM CHLORIDE OVER A FIVE-DAY PERIOD

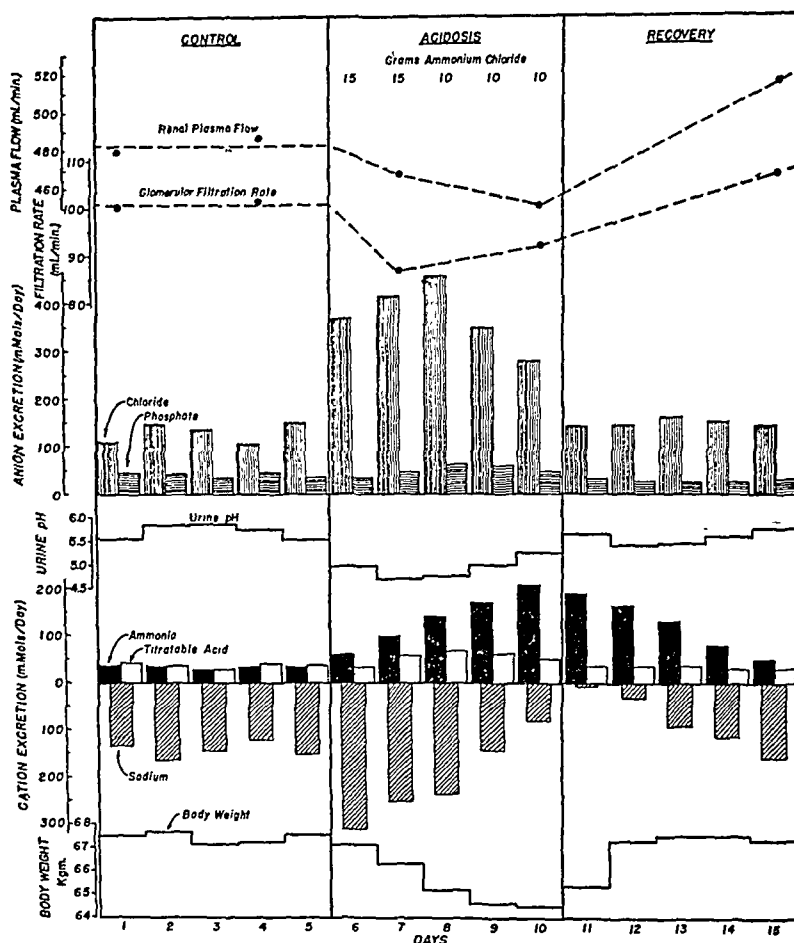


FIG. 2. CHANGES IN THE RATES OF EXCRETION OF IONS, RENAL PLASMA FLOW, GLOMERULAR FILTRATION RATE, AND BODY WEIGHT WHICH RESULTED FROM THE INGESTION OF 60 GMS. OF AMMONIUM CHLORIDE OVER A FIVE-DAY PERIOD

remaining three days. The second subject ingested 15 gms. daily throughout the period of acidosis.

(b) Renal clearance experiments were performed at intervals during the control, acidosis, and recovery periods. In the first subject, the rate of glomerular filtration was approximated by mannitol clearances,³ performed on the first and fourth days of the control period, the second and fifth days of acidosis and the fifth day of recovery. In the second subject, inulin was used as an exact measure of glomerular filtration rate. Determinations were made on the first and fourth days of each of

³ According to recent studies of Berger *et al.* (23) the mannitol clearance is significantly lower than the inulin clearance, hence is not an exact measure of glomerular filtration rate. However, the mannitol clearance presumably measures a constant fraction of the glomerular clearance in any one individual. Thus although absolute values for ionic reabsorption calculated from mannitol clearances may be too low, changes in ionic reabsorption are correctly indicated.

the three experimental periods. Effective renal plasma flow was determined by the *p*-amino-hippurate clearance in both subjects.

(c) The acute response of the kidney to acidosis was assessed in one subject in a 12 period experiment, during which a total of 10 gms. of ammonium chloride was ingested.

RESULTS

Acid-base equilibria of plasma and urinary ionic balances in acidosis

Data on the plasma composition and urinary ionic balance of subject P are summarized in Figures 1 and 2. In Figure 1 are presented the plasma values for chloride, bicarbonate, $p\text{CO}_2$, pH, sodium and phosphate. Each variable is referred to a base line established by averaging the five control determinations. Thus the mean

plasma chloride during the control period was 107 mEq per liter, varying within limits of 105 and 109 mEq per liter. In consequence of ingestion of ammonium chloride, the plasma chloride rose to a peak value of 122.8 mEq per liter on the fourth day of acidosis. In contrast the plasma bicarbonate fell from a mean normal value of 24.4 mEq per liter to 10.3 mEq per liter on that day.

Chloride and bicarbonate varied throughout in reciprocal fashion, the sum of the two remaining roughly constant for the first 11 days of the experiment. However, on the second recovery day, the sum of these two anions increased 6 mEq per liter, for bicarbonate rose more rapidly than chloride fell during this period. The sum of chloride and bicarbonate averaged 7 mEq lower than sodium during the control period and at the end of the recovery period. However, during the period of acidosis, sodium fell 2 to 3 mEq below the sum of these two anions. This finding can be partially explained by the decrease in base binding capacity of the plasma protein due to increased plasma acidity. However, this and other interesting results prompted a more complete ionic study of the plasma and urine in a second subject.

Respiratory compensation is shown by the drop in $p\text{CO}_2$ of arterialized venous blood from a normal value of 41.0 to 23.9 mm. Hg during acidosis; the latter value is indicative of a 70 per cent increase in alveolar ventilation over the normal. The subject was conscious of some increased ventilation at rest and obvious dyspnea on exertion. Presumably this increase in ventilation resulted from the increase in blood acidity from pH 7.40 to 7.25 (24).

The serum sodium concentration dropped significantly on the second day of acidosis and reached a low of 130.9 mEq per liter on the third day, a decrease of 8 mEq per liter. The plasma concentration of phosphate, in contrast, was maintained relatively constant until the fifth day of acidosis.

The concentrations of each of these plasma constituents with the exception of phosphate returned to normal or supra-normal levels within the five-day recovery period. Serum sodium and pH were restored first, bicarbonate and $p\text{CO}_2$ next, and chloride only on the fifth day. There

is evident an over-compensation in the return of bicarbonate, $p\text{CO}_2$, pH, and sodium to levels above those existing during the control periods.

In Figure 2 are summarized the rates of excretion of chloride, phosphate, titratable acid, ammonia and sodium, expressed in mEq per 24 hours; urine pH; body weight; and glomerular filtration rate and renal plasma flow on the five days on which these variables were measured. During the control period the rate of excretion of chloride averaged 130 mEq per day varying within limits of 107 and 151 mEq per day. In consequence of the ingestion of ammonium chloride and elevation of plasma level, chloride excretion increased to 368 mEq on the first day and to 458 on the third. During these three days there was a net loss of 230 mEq of chloride over and above that ingested in the diet and as ammonium chloride. At a mean concentration of 115 mEq per liter, this represents a loss of 2 liters of extracellular fluid in the first three days of acidosis. Body weight actually decreased 2.2 Kgm. during this interval. Although a rigorous analysis of these data cannot be justified, it is safe to infer that the major loss of weight early in this experiment resulted from loss of extracellular fluid.

The excretion of phosphate increased from an average of 41 mM per day during the control period to a peak of 60 mM per day on the third day of acidosis. Phosphaturia was not as significant a feature of acidosis in these experiments as in some previously reported (1). However, it accounted in large measure for the increased excretion of titratable acid from the mean control rate of 37 mEq per day to a peak of 68 mEq per day. The fall in urine pH from a control value of 5.7 to a low of 4.7 contributed, although less significantly than phosphate excretion, to the increased excretion of acid. Since increased excretion during the first four days of acidosis was attended by no fall in plasma level, phosphate must have been derived either from skeletal or intracellular stores. The fact that in subject H both calcium and potassium excretion were elevated during acidosis inclines us to the view that phosphate was derived from both possible sources.

The well known excretion of fixed base at the onset of acidosis, and its subsequent replacement with ammonia are well illustrated in this experi-

ment. During the control period, 142 mEq of sodium and 34 mEq of ammonia were excreted per day. On the first day of acidosis the excess chloride in the urine was largely neutralized with sodium, the rate of excretion of this latter ion rising to 310 mEq per day, whereas that of ammonia increased only to 62 mEq per day. By the fifth day ammonia had increased sufficiently to neutralize all excess chloride derived from the ammonium salt ingested, and the sodium balance became positive. On the first day of the recovery period the retention of dietary sodium was nearly complete, most of the urinary anions being neutralized by ammonia. A positive sodium balance was maintained for four days, during which time the alkali reserve and the salt content and volume of the several fluid compartments of the body were restored to their original condition.

During the five-day period of acidosis the net loss of sodium from the body amounted to 306 mEq. At a mean plasma sodium concentration of 135 mEq per liter this represents a loss of 2.3 liters of extracellular fluid. Actually body weight decreased during this interval by 2.9 Kgm. The extra weight loss probably represents intracellular fluid, for in subject H a significant loss of potassium was observed during the period of acidosis. It is, however, apparent that in this experiment roughly three-fourths of the weight loss can be ascribed to a reduction in volume of extracellular fluid. It is significant that in the first four days of recovery 309 mEq of sodium were retained to balance the 306 mEq lost during the period of acidosis. On the fifth day of recovery sodium excretion was exactly equal to the average of the five control days. Before this time body weight had been restored to normal.

A part of the fluid lost during the period of acidosis was derived from the vascular compartment, for the hematocrit, which fell steadily during the five control days, rose during the first three days of acidosis to a value appreciably above normal, to fall precipitously during the recovery period. In all some 1300 ml. of blood were drawn, relatively equally distributed over the 15 days of the experiment, so that a steady fall in hematocrit would be expected had no blood concentration resulted. No doubt because of this reduction in blood volume, glomerular filtration rate and renal plasma flow fell from their respec-

tive control values of 101 and 483 cc. per min. to 86.8 and 468 cc. per min. on the second day of acidosis, and to 92 and 402 cc. per min. on the fifth day. These changes in renal function are rather small, but had the dehydration been more severe and progressive it is probable that they would have been of greater magnitude. It must be remembered that the intake of food, fluid and salt was maintained at normal levels throughout the period of developing acidosis, a fact which must have reduced the severity of dehydration in comparison with that observed in patients suffering from diabetic ketosis or chronic nephritis, and exhibiting equivalent reduction in plasma alkali reserve. At the end of the recovery period both filtration rate and renal plasma flow had returned to levels slightly higher than those of the control period. Subject H who lost less weight and exhibited less dehydration suffered no decrease in filtration rate or in renal plasma flow; indeed both variables increased somewhat during acidosis. It is reasonable to infer that the dehydration which may accompany acidosis rather than the acidosis itself is the cause of the reduced renal function often observed (8, 25).

The results of the more extensive balance experiment performed on Subject H are summarized in Figures 3-5. In Figure 3 the plasma values for sodium, potassium, and calcium are presented in the left hand block, and those for chloride, bicarbonate, protein, phosphate, and sulphate are presented in the right hand block of each day's result. An undetermined moiety remains in the cation column which increases during acidosis from a control average of 6.2 mEq per liter to 10.0 mEq per liter on the last day of acidosis. The undetermined moiety is no doubt in part magnesium, and if one accepts a figure of 4 mEq per liter as a normal plasma value, the discrepancy during the control period is reduced to a satisfactorily small value. It must be remembered that the protein equivalence is calculated from total protein of serum and may well be subject to error. An increase in this error during acidosis may account for the additional slight discrepancy. On the other hand, as noted below, it may be in part due to an increase in plasma magnesium. During the control period total plasma anions average 158.2 mEq per liter. There occurred a slight decrease during acidosis to an

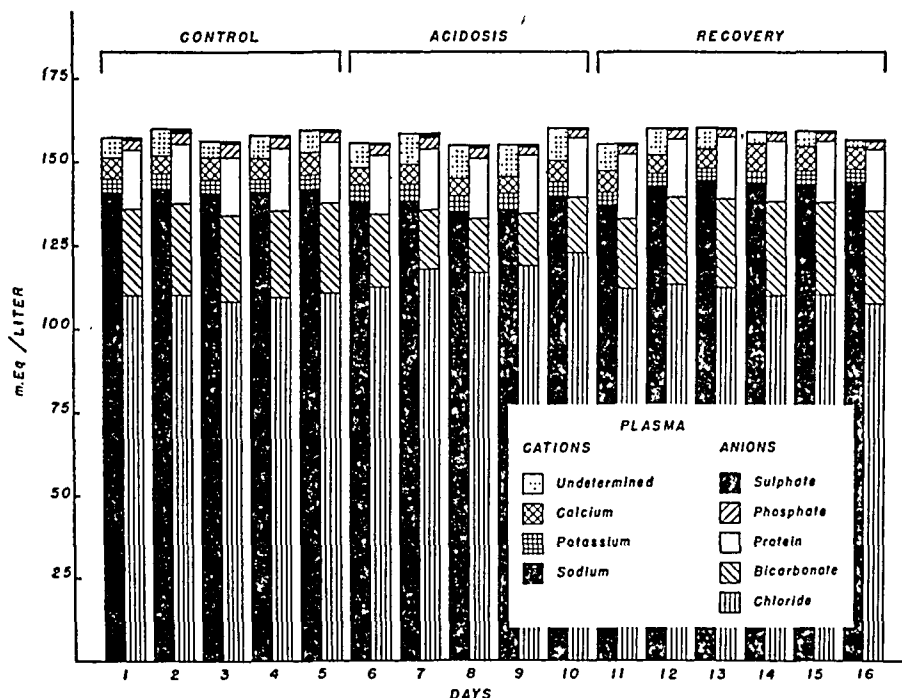


FIG. 3. CHANGES IN THE ELECTROLYTE PATTERN OF THE PLASMA OF SUBJECT H WHICH RESULTED FROM THE INGESTION OF 75 GMS. OF AMMONIUM CHLORIDE OVER A FIVE-DAY PERIOD

average of 156.9, rising again in recovery to 158.6 mEq per liter. Plasma sodium dropped from a control average of 141.2 mEq per liter to a low of 135.3 on the third day of acidosis, a decrease of 5.9 mEq per liter. This decrease is slightly less than that observed in subject P. Chloride, in consequence of the ingestion of ammonium chloride, rose from a control average of 109.8 mEq per liter to a peak value of 123.1 on the last day of acidosis, a rise of 13.2 mEq per liter. Conversely bicarbonate fell during acidosis from a mean control level of 26.3 mEq per liter to a low of 15.6 mEq per liter on the fourth day. Again as in subject P, the sum of chloride and bicarbonate remained roughly constant from day to day until the second recovery day when a moderate rise occurred due to the rapid return of bicarbonate to normal. The mean control value for the sum of chloride and bicarbonate was 136.1 mEq per liter, a value 5.1 mEq per liter lower than the control sodium level. This difference decreased during acidosis so that on the last day of this period it amounted only to 0.4 mEq per liter. As may be seen from Figure 3, this change in the proportion of sodium to the sum of chloride and bicarbonate may be ascribed in part to the slight increase in plasma potassium from a mean

control level of 4.26 mEq per liter to a high of 4.95 mEq per liter during acidosis, to the small reduction in phosphate from a control of 2.6 mEq per liter to a low of 2.1 mEq per liter on the last day of acidosis, and to the minor decrease in sulphate from an average of 1.1 mEq per liter to a low of 0.8 mEq per liter again on the last day of acidosis. The more significant change appears in the undetermined moiety and may represent some increase in plasma magnesium and possibly an error in the base binding power of the plasma proteins as noted above. Further investigation is needed to clarify this point. However, both subjects exhibited the same type of change in sodium/anion ratio to approximately the same degree.

The plasma pH averaged 7.41 during the control period and fell progressively during the period of acidosis to a low of 7.29, only to rise rapidly to a level of 7.41 on the second recovery day. An over-compensation then resulted with the pH increasing to 7.45 on the fourth and fifth recovery days. A similar over-compensation was observed during the recovery period with respect to sodium, calcium and bicarbonate levels.

Electrolyte excretion is graphically presented in Figure 4. Cation excretion is blocked against anion excretion for each day of the experiment.

All values are given in milliequivalents per 24 hours. In consequence of the ingestion of ammonium chloride, total ionic excretion (sum of anions and cations) rose steadily during the period of acidosis so that on the last day of this period a total of 1248 mEq per 24 hours were excreted as compared to a mean control value of 566 mEq. During the control period, the mean daily chloride excretion was 9 mEq higher than the mean sodium excretion, their values being 134.5 and 125.0 mEq per 24 hours respectively. However, with the ingestion of 15 gms. of ammonium chloride daily, chloride excretion rose progressively over the five-day period of acidosis to 470 mEq per 24 hours. Sodium excretion was greatest on the first day of acidosis and then fell in stepwise fashion from 293 to 50.6 mEq per 24 hours on the second day of recovery. Potassium excretion averaged 79.8 mEq per 24 hours during the control period, rose to a peak of 166.6 mEq per 24 hours on the third day of acidosis, and then fell gradually to a low of 20.9 mEq per 24 hours on the second recovery day. It is interesting that potassium excretion had not risen appreciably by the last day of recovery even though the net deficit incurred during the period of acidosis had been almost completely regained. Calcium

excretion rose to a high value of 41.4 mEq per 24 hours on the fourth day of acidosis from a mean control value of 17.6 mEq per 24 hours. In contrast to potassium excretion, the excretion of calcium remained above the control value during the recovery period, averaging 27.7 mEq for the six days of this period and falling to 24.8 mEq per 24 hours on the last day of the experiment. Magnesium excretion varied but little from a mean control level of 13.1 mEq per 24 hours reaching a maximum of only 16.8 mEq on the second day of acidosis and falling in the recovery period to a level only slightly below the control. With the drop in urine pH from a mean control level of 5.61 to an average of 4.92 during the period of acidosis, the capacity of creatinine to combine with acid became significant. Thus acid bound by creatinine rose from a mean control value of 3.8 mEq per 24 hours to a high of 10.1 mEq per 24 hours on the second day of acidosis. Sulphate excretion averaged 49.4 mEq per 24 hours during the control period, rising to 63.6 on the second day of acidosis and dropping to a low of 22.6 mEq per 24 hours on the first day of recovery. Phosphate excretion per 24 hours averaged several mEq greater than sulphate but behaved in a similar way. Organic acid excretion showed no

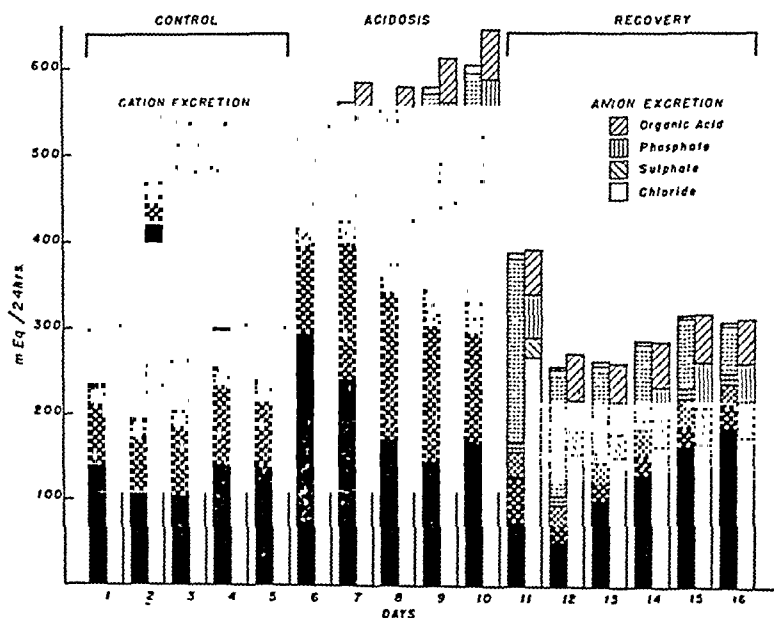


FIG. 4. CHANGES IN THE DAILY EXCRETION OF IONS OF SUBJECT H RESULTING FROM THE INGESTION OF 75 GMS. OF AMMONIUM CHLORIDE OVER A FIVE-DAY PERIOD

significant change during the experiment. Discrepancies between anion and cation excretion during the control and recovery periods are within limits of experimental error. During acidosis the increased discrepancy is unexplained, but might be due in part to the elimination of some undetermined organic cation, *e.g.*, a basic amino acid such as histidine.

In the central portion of Figure 5, base conservation, as represented by the excretion of ammonia and titratable acid, is plotted upwards while fixed base loss is plotted downwards. Again, it may be clearly seen that on the first day of acidosis the excess chloride in the urine was to a large extent neutralized by sodium. On the second and succeeding days, sodium excretion was spared not only by an increase in ammonia and titratable acid excretion but also by an increase in the excretion of potassium and calcium. During the last two days of acidosis and the early days of recovery, ammonia and titratable acid excretion

were sufficiently great to permit a progressive and significant decrease in all elements comprising fixed base. Attention is called to the gradual return of ammonia to the control level, for in this phenomenon lies the explanation of the restoration and over-expansion of the base reserves during recovery.

Daily urine output is graphed on an inverted scale immediately below fixed base loss in Figure 5. The increase in output during the period of acidosis is in large part the result of the increased intake of water to dilute the ammonium chloride ingested, for body weight decreased only by 1.4 Kgm. during the entire interval. Calculations of net loss of intra- and extracellular fluid during acidosis were less satisfactory in subject H than in subject P, because of less adequate dietary control and especially because of the relatively small change in body weight. Judging from the excess sodium and potassium excretion, loss of fluid was relatively equally distributed between the two compartments. Evidence of reduction in circulating blood volume either from plasma proteins or hematocrit such as that seen in subject P was nil. Accordingly no fall, and indeed an increase in glomerular filtration rate and renal plasma flow was observed. These increases are unexplained though they may be within limits of daily variation in this subject.

Ionic reabsorption in acidosis

It is obvious from a comparison of Figures 1 and 2, and of Figures 3, 4, and 5 that the increase in the excretion of chloride during ammonium chloride acidosis is related to the increase in plasma chloride concentration, and that the increase in excretion of sodium is the causative factor in the decrease in plasma sodium concentration and in the decline in body weight. Experiments in which glomerular filtration rate was measured permit a more exact description of the changes in renal function which underlie the altered excretion of the several ions. The significant data derived from 11 experiments performed at regular intervals on the two subjects during the control, acidosis, and recovery periods, are summarized in Tables I and II. Inasmuch as filtration rate decreased in one subject and increased in the second during acidosis, and since

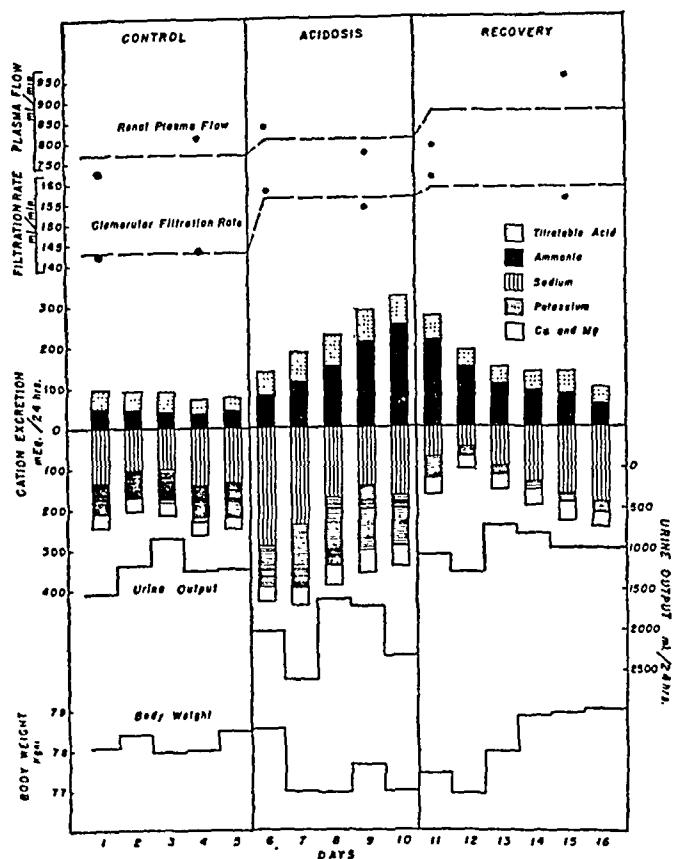


FIG. 5. CHANGES IN THE EXCRETION OF CATIONS, RENAL PLASMA FLOW, GLOMERULAR FILTRATION RATE, URINE OUTPUT, AND BODY WEIGHT IN SUBJECT H RESULTING FROM THE INGESTION OF 75 GMS. OF AMMONIUM CHLORIDE OVER A FIVE-DAY PERIOD

RENAL COMPENSATIONS IN AMMONIUM CHLORIDE ACIDOSIS

TABLE I
Rates of reabsorption and excretion of ions in subject P determined on the first and fourth days of the control periods, on the second and fifth days of ammonium chloride acidosis, and on the fifth day of recovery

TABLE 1 Rates of reabsorption and excretion of ions in subject P determined on the first and fourth days of the ammonium chloride acidosis, and on the fifth day of recovery																
Urine flow	Glomer- ular filtration rate	Plasma						Excreted					Reabsorbed			
		Sodium	Bicar- bonate	Chlo- ride	Phos- phate	Sodium	Bicar- bonate	Chloride	Phos- phate	Ammo- nia	Titratable acid	Sodium	Bicar- bonate	Chloride	Phos- phate	
ml./min.	ml./min.	mEq/l.	mEq/l.	mM/l.	mEq/100 ml. glomer. filt.	mEq/100 ml. glomer. filt.	mEq/100 ml. glomer. filt.	mEq/100 ml. glomer. filt.	mM/min.	mEq/min.	mEq/min.	mEq/100 ml. glomer. filt.	mEq/100 ml. glomer. filt.	mEq/100 ml. glomer. filt.	mM/min.	
Control Day 1	2.95	100.0	138.2	24.2	107.2	1.26	0.34	.000	0.32	0.009	0.030	0.014	13.48	2.42	10.40	0.117
	3.60	103.4	137.5	24.5	106.7	1.25	0.35	.000	0.35	0.013	0.013	0.015	13.40	2.45	10.32	0.116
	3.05	96.8	138.3	24.0	106.2	1.26	0.31	.000	0.29	0.013	0.032	0.018	13.52	2.40	10.33	0.109
	3.20	100.1	138.0	24.2	106.7	1.26	0.33	.000	0.32	0.012	0.031	0.016	13.47	2.43	10.35	0.114
Control Day 4	3.00	98.2	137.5	24.4	104.4	1.10	0.26	.000	0.27	0.007	0.026	0.011	13.49	2.44	10.17	0.101
	2.85	98.2	137.6	24.1	104.4	1.15	0.24	.000	0.27	0.007	0.025	0.011	13.52	2.41	10.17	0.106
	3.25	108.3	137.4	23.6	106.1	1.16	0.26	.000	0.29	0.008	0.030	0.012	13.48	2.36	10.32	0.108
	3.03	101.6	137.5	24.0	105.0	1.14	0.25	.000	0.69	0.026	0.027	0.011	13.50	2.40	10.88	0.093
Acidosis Day 7	3.95	84.7	133.3	14.2	115.7	1.40	0.49	.000	0.63	0.026	0.074	0.045	12.84	1.42	10.94	0.092
	3.65	88.4	133.3	13.5	115.8	1.35	0.43	.000	0.64	0.027	0.076	0.045	12.90	1.35	10.96	0.091
	3.40	87.2	133.1	13.4	115.9	1.41	0.41	.000	0.65	0.032	0.079	0.045	12.90	1.34	10.93	0.092
	3.67	86.8	133.2	13.7	115.8	1.39	0.45	.000	0.50	0.028	0.076	0.045	12.88	1.37	10.93	0.092
Acidosis Day 10	3.05	93.9	130.8	11.8	117.5	0.998	0.30	.000	0.49	0.026	0.144	0.040	12.78	1.18	11.25	0.068
	2.95	91.0	131.9	11.8	117.5	0.996	0.29	.000	0.47	0.026	0.143	0.040	12.90	1.18	11.26	0.065
	2.85	91.0	128.8	11.8	116.6	1.04	0.27	.000	0.49	0.027	0.143	0.040	12.61	1.18	11.19	0.068
	2.95	92.0	130.5	11.8	117.2	1.011	0.29	.000	0.49	0.026	0.143	0.040	12.76	1.18	11.23	0.067
Recovery Day 15	2.45	107.2	141.0	28.0	106.8	1.01	0.28	.000	0.28	0.004	0.038	0.008	13.82	2.80	10.40	0.106
	2.60	102.0	139.8	27.8	107.1	1.04	0.31	.000	0.32	0.005	0.040	0.008	13.67	2.78	10.39	0.108
	2.65	111.5	138.6	27.8	106.3	0.99	0.35	.000	0.32	0.007	0.041	0.008	13.51	2.78	10.31	0.097
	2.57	106.9	139.9	27.9	106.7	1.01	0.31	.000	0.31	0.005	0.040	0.008	13.67	2.79	10.37	0.104

previous work has shown that the quantities of sodium, bicarbonate, and chloride reabsorbed vary in proportion to glomerular filtration rate (22, 26, 27), the reabsorption and excretion of these ions have been expressed in mEq per 100 ml. of glomerular filtrate for comparative purposes. On the other hand, in Table I, the reabsorption and excretion of phosphate have been expressed in mM per min., for the capacity of the kidney to reabsorb this ion is fixed, limited and independent of filtration rate (28). The rates of excretion of titratable acid and ammonia are likewise expressed in mEq per min., for both hydrogen ions and ammonia are eliminated by mechanisms which are basically secretory in character, hence not related to filtration rate (29).

In Table I, it may be seen that in the two control experiments at a plasma concentration of 138 mEq per liter, 13.5 mEq of sodium were reabsorbed and 0.3 mEq were excreted per 100 ml. of glomerular filtrate. In the first experiment in acidosis although the quantity of sodium delivered into the renal tubules per unit volume of filtrate was less in consequence of the drop in plasma concentration, that lesser quantity was reabsorbed less completely than under normal conditions. Excretion rose to 0.45 mEq and reabsorption decreased to 12.9 mEq per 100 ml. of filtrate. Indeed decreased reabsorption and increased excretion must have been the major determinants of reduced plasma concentration and of loss of sodium from the body. By the fifth day of acidosis, the urinary loss of sodium had diminished to the control level, namely 0.29 mEq per 100 ml. of filtrate. No less than three factors contributed to diminished sodium loss: low plasma sodium concentration, *i.e.*, reduced sodium content of the filtrate; diminished chloride load demanding excretion, *i.e.*, intake of ammonium chloride had been cut from 15 to 10 gms. per day; and of greatest significance, increased substitution of ammonia for sodium in neutralizing urinary anions. We interpret the initial loss of sodium as the passive consequence of the increased excretion of chloride, brought about by the increased chloride load presented to the tubules. However, the reabsorption of chloride must be considered in relation to the reabsorption of bicarbonate, for previous work has shown that the mechanisms responsible for the salvage of these ions are inter-

related in such a way that decreased reabsorption of one ion species is *partially* compensated by increased reabsorption of the other (22). Since all urines were more acid than pH 6.0 the excretion of bicarbonate was negligible throughout the entire series of experiments. Because of reduced plasma concentration, less bicarbonate per unit volume of filtrate was presented for reabsorption in the two experiments in acidosis than in the two controls. Accordingly greater quantities of chloride were reabsorbed in acidosis than normally, *e.g.*, 10.9 and 11.2 mEq per 100 ml. of glomerular filtrate in acidosis, in comparison with 10.4 and 10.2 mEq normally. Yet because of the greatly increased load of chloride, even the enhanced reabsorptive capacity was exceeded, and chloride excretion rose. Over-compensation during the recovery period is evident in the reabsorption of a greater than normal quantity of bicarbonate, the reabsorption of chloride reverting more or less to the control value.

Diminished absorption and increased excretion of sodium during acidosis with compensatory over-absorption and diminished excretion during recovery are again evident in experiments on subject H presented in Table II. Thus in the second control experiments 13.71 mEq of sodium per 100 ml. of filtrate were reabsorbed and 0.12 mEq excreted. On the first day of acidosis 13.60 mEq were reabsorbed and 0.25 mEq were excreted. On the first day of recovery reabsorption had increased to 13.78 mEq and excretion had fallen to 0.036 mEq. These days are chosen for consideration because the plasma sodium concentrations were essentially the same on all. Such changes in absorption and excretion are small, but it is upon such small changes that the negative sodium balance early in acidosis and the positive sodium balance in recovery depend. For instance, a decrease in the absorption of sodium of 0.11 mEq per 100 ml. of filtrate would cause the loss of 250 mEq in a day's time at the filtration rate exhibited by subject H on the first day of acidosis. This would be equivalent to the loss of 1.8 Kgm. of extracellular fluid. The actual negative balance on this day was 168 mEq of sodium rather than the 250 mEq calculated, and the loss of weight was 1.4 Kgm. rather than 1.8 Kgm. One must infer either that the diminished absorption noted above was not maintained throughout

TABLE II
Rates of reabsorption and excretion of ions in subject H. on the first and fourth days of each of the three experimental periods

	Urine flow	Plasma			Excreted				Reabsorbed			
		Sodium	Potassium	Chloride	Sodium	Potassium	Chloride	Ammonia	Titratable acid	Sodium	Potassium	Chloride
Control Day 1	ml./min.	mEq/l.	mEq/l.	mEq/l.	mEq/100 ml. glomer. fil.	mEq/100 ml. glomer. fil.	mEq/100 ml. glomer. fil.	mEq/min.	mEq/min.	mEq/100 ml. glomer. fil.	mEq/100 ml. glomer. fil.	mEq/100 ml. glomer. fil.
	1.74	140.0	4.47	111.2	0.156	0.046	0.063	0.024	0.042	13.84	0.401	11.06
	3.90	140.0	4.36	110.7	0.161	0.051	0.152	0.052	0.040	13.84	0.385	10.92
	2.55	139.9	4.41	110.8	0.184	0.054	0.164	0.044	0.041	13.81	0.387	10.92
Control Day 4	2.73	139.8	4.41	110.9	0.167	0.050	0.126	0.040	0.041	13.83	0.363	10.97
	10.45	138.5	4.12	108.2	0.109	0.049	0.085	0.033	0.019	13.74	0.363	10.74
	2.45	138.2	4.13	107.9	0.121	0.041	0.104	0.027	0.019	13.70	0.372	10.69
	6.25	138.0	4.03	108.7	0.117	0.041	0.097	0.047	0.030	13.68	0.362	10.77
Acidosis Day 6	6.38	138.2	4.09	108.3	0.116	0.043	0.095	0.036	0.023	13.71	0.366	10.73
	12.70	138.1	4.68	111.4	0.229	0.095	0.308	0.089	0.064	13.58	0.373	10.83
	6.80	138.7	4.77	112.0	0.259	0.087	0.342	0.078	0.064	13.61	0.390	10.86
	7.55	138.9	4.66	111.7	0.272	0.091	0.344	0.085	0.064	13.62	0.375	10.83
Acidosis Day 9	9.02	138.6	4.70	111.7	0.253	0.091	0.331	0.084	0.064	13.60	0.379	10.84
	10.20	135.8	4.57	117.6	0.164	0.131	0.338	0.187	0.060	13.42	0.326	11.42
	10.80	137.1	4.33	118.0	0.197	0.129	0.370	0.183	0.059	13.51	0.304	11.43
	8.45	135.3	4.32	118.4	0.168	0.115	0.328	0.192	0.061	13.36	0.317	11.51
Recovery Day 11	9.82	136.1	4.41	118.0	0.176	0.125	0.345	0.187	0.060	13.43	0.316	11.45
	0.90	137.3	3.75	112.7	0.033	0.014	0.084	0.163	0.030	13.70	0.361	11.19
	1.35	138.4	3.59	112.5	0.049	0.026	0.086	0.169	0.030	13.79	0.333	11.16
	5.55	138.9	3.57	112.8	0.027	0.020	0.069	0.157	0.028	13.86	0.337	11.21
Recovery Day 14	2.60	138.2	3.64	112.7	0.036	0.020	0.080	0.163	0.029	13.78	0.344	11.19
	1.65	142.2	3.46	109.2	0.096	0.011	0.090	0.091	0.023	14.12	0.335	10.83
	2.95	142.5	3.54	109.8	0.105	0.012	0.092	0.086	0.025	14.15	0.342	10.69
	2.80	143.1	3.52	109.3	0.113	0.012	0.105	0.075	0.028	14.20	0.340	10.83
	2.47	142.6	3.51	109.4	0.105	0.012	0.096	0.084	0.025	14.16	0.339	10.78

the 24 hours or that the filtration rate was lower at night (a reasonable assumption [30]). On the first day of recovery the increase in the absorption of sodium of 0.07 mEq per 100 ml. of filtrate over the last control period would cause the retention of 161 mEq in 24 hours and a gain of 1.2 Kgm. in weight. This quantity of sodium was not available in the diet and the actual sodium retention and weight gain were only one-third of the calculated values. A comparison of Tables I and II indicates that the greater sodium deficit, greater loss of weight and more significant fall in plasma sodium level in subject P as compared with subject H was the consequence of a greater increase in sodium excretion.

As shown in Table II potassium excretion increased sharply during acidosis, rising from a control average of 0.047 mEq per 100 ml. of glomerular filtrate to 0.091 mEq on the first day of acidosis and to 0.125 mEq on the fourth day of acidosis. On this latter day at the same plasma level as on the first control day, it is evident that the absorption per 100 ml. of filtrate was significantly diminished. Despite diminished absorption and increased excretion, plasma potassium was maintained at a nearly constant level, a fact explicable only on the assumption that the excess potassium was derived from cellular stores. Loss of intracellular potassium in acidosis has been adequately demonstrated (31). During recovery, potassium excretion dropped sharply to 0.020 mEq per 100 ml. of filtrate on the first day and to 0.012 mEq on the fourth day. This lower excretory rate during recovery must have been conditioned in part by the low plasma potassium level. In turn this low plasma level must have been caused by the restoration of depleted cellular reserves from the ingested and circulating moiety.

Depressed absorption of phosphate in acidosis is evident from the data of Table I. In the first experiment in acidosis 24 per cent of the filtered phosphate was excreted at a plasma concentration of 1.4 mEq per liter; in the second, 30 per cent was excreted at a significantly lower plasma concentration. In the control periods and following recovery only 5 to 10 per cent of the filtered phosphate was excreted. These results are in line with previous findings in man, namely, that acidosis depresses the tubular reabsorption of phosphate at normal plasma levels, although it

does not affect maximum tubular reabsorptive capacity at high plasma levels (32). The reduced plasma level of phosphate during recovery, like that of potassium, must have resulted from diversion of phosphate from plasma to cellular and osseous depots depleted during acidosis.

Early time course of renal compensations in acidosis

In order to study the time course of early renal compensations in acidosis a 12 period experiment was performed in which plasma and urine samples were collected at 15 min. intervals. During this experiment a total of 10 gms. of ammonium chloride was ingested in two doses. The significant observations are presented in Figure 6. The first two periods, performed with the subject in a state of normal acid-base balance, established control values for all variables. A 5 gm. dose of ammonium chloride was then ingested and following four additional clearance periods, another 5 gms. were taken.

Minimal changes in plasma chloride, bicarbonate and pH are evident within 7.5 min. after the ingestion of the first dose of ammonium chloride.

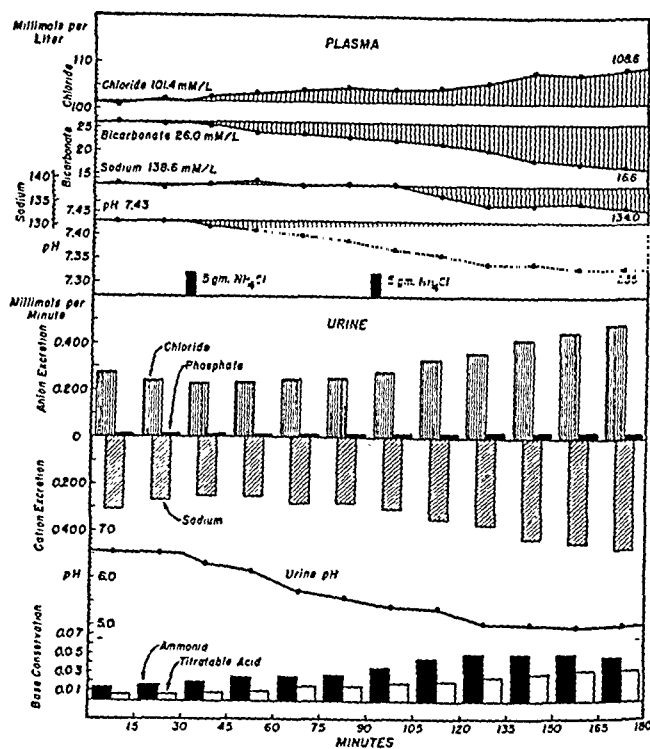


FIG. 6. CHANGES IN IONIC COMPOSITION OF THE PLASMA, IN RATES OF EXCRETION OF IONS DURING THE ACUTE PHASE OF A DEVELOPING AMMONIUM CHLORIDE ACIDOSIS

These changes became progressively more significant over the succeeding 45 min. Following the second dose, plasma chloride rose and bicarbonate, pH and sodium fell within the subsequent 90 min. to levels characteristic of moderately severe acidosis. A decrease in pH and an increase in content of titratable acid and ammonia are evident in the urine formed within 15 min. following the ingestion of the first dose of the acidifying salt, changes which were progressive throughout the course of the experiment. No significant changes in the excretion of phosphate, chloride and sodium were evident until the second 5 gms. of ammonium chloride were ingested, although plasma chloride rose appreciably during the interval between the two doses, a rise which was exactly counterbalanced by the fall in plasma bicarbonate. But following the second dose of the ammonium salt, chloride excretion increased, and since the excess urinary chloride was largely neutralized by sodium, the excretion of this ion increased proportionately. Only a part of the reduction in plasma sodium can be ascribed to increased excretion; the remainder must have been due to shifts of fluid and/or electrolyte between intra- and extracellular compartments.

One surprising finding in this experiment, which has been observed in others as well, was the prompt increase in the excretion of ammonia. It is usually stated that the kidney responds early to an increased acid load by excreting acid in free titratable form, and only after some delay, by excreting increased quantities of ammonia. It is evident from Figure 3 that ammonia and titratable acid excretion increased at equivalent rates and to approximately the same extent within the first 120 min. after the ingestion of an acidifying salt. It is true that this increase in ammonia output is only a small fraction of that attained after five days, but there certainly is no true delay in its onset, merely a gradual attainment of its maximal value. If it is correct to infer that no significant increase in plasma ammonia results from the ingestion of ammonium chloride (1, 6), then the observed increase in the rate of ammonia excretion constitutes a prompt renal compensation to ingestion of acid. Since plasma phosphate level did not change, the increased rate of excretion of phosphate during the latter half of this experiment resulted from diminished reabsorption. Both

the increase in the rate of phosphate excretion and the increase in the acidity of the urine contributed to the enhancement of titratable acid excretion.

DISCUSSION

It is apparent in ammonium chloride acidosis that the kidney maintains approximate constancy of total ionic concentration at the expense of ionic pattern and to a lesser degree at the expense of extracellular fluid volume. As bicarbonate is converted to chloride an increase in the tubular reabsorption of this latter ion species serves to restrict its loss, and thus the loss of base which accompanies it into the urine. For example, 10.3 mEq of chloride were reabsorbed and 0.28 mEq were excreted per 100 ml. of glomerular filtrate in the second control experiment presented in Table I. On the first day of acidosis increased quantities of chloride per unit volume of filtrate were delivered into the renal tubules, because of increased plasma concentration. However, an increase in reabsorption to 10.93 mEq per 100 ml. of filtrate limited excretion to 0.65 mEq. Had no increase in chloride absorption occurred, excretion would have increased to 1.36 mEq and the rate of sodium loss would have been tripled. It is apparent that an important renal response in ammonium chloride acidosis is the increase in tubular reabsorption of chloride, an increase which is more or less proportional to the extent of the conversion of bicarbonate to chloride. A consequence of this renal response is the maintenance of constancy of the sum of the plasma concentrations of chloride and bicarbonate despite considerable shifts in their relative proportions.

However, the conversion of a significant proportion of bicarbonate to chloride is attended by some increase in chloride excretion. Early in acidosis the excess urinary chloride is neutralized in large part by sodium derived from extracellular stores. This loss of fixed base is accompanied by the excretion of nearly equivalent quantities of water. Since the specific gravity of the urine of subject P averaged 1.016 during the five days of acidosis in contrast to 1.026 during the five control days, it is probable that the polyuria and consequent loss of weight during acidosis resulted from diminished posterior pituitary activity rather than from osmotic abstraction of body water by

concentrated urinary solutes. Indeed the probable stimulus promoting fluid elimination was the decrease in osmotic pressure of the body fluids in consequence of base loss. This is suggested by the fact that water excretion lagged somewhat behind base loss, for in both subjects plasma sodium level decreased significantly.

In subject P a major portion (three quarters) of the water and base sacrificed during acidosis was derived from the extracellular fluid compartment (*cf.* calculations on p. 426). In subject H balances were less accurate and calculations less dependable, but fluid loss seemed more equally divided between intra- and extracellular compartments. It is significant in subject H that the majority of the base lost on the first day of acidosis was sodium. However, by the third day potassium excretion had increased to a point where this ion neutralized a greater proportion of the urinary acid than did sodium. Since the plasma level of potassium did not change appreciably during acidosis, this ion must have been delivered into the blood from the tissues at essentially the same rate at which it was eliminated in the urine. The substitution of potassium for sodium in the urine permits the body to draw on the large intracellular reserves of buffer base; hence loss of interstitial fluid and reduction of circulating plasma volume are curtailed.

The changes in renal function described above are restrictive compensations, in the sense that they limit the disturbance of total ionic concentrations and volumes of both intra- and extracellular fluids. The elimination of titratable acid and especially of ammonia are corrective compensations in the sense that they permit the continued excretion of acid without further drain of base, and during recovery, rapidly restore ionic patterns, total ionic concentrations and volumes to normal. It is apparent from Figures 2 and 4 that ammonia excretion and to a lesser extent titratable acid excretion increased progressively over the five-day period of acidosis. In subject P, who ingested a smaller dose of ammonium chloride, the ammonia output on the fifth day was more than equivalent to the acid load, and a positive sodium balance was attained. In subject H, the ammonia output was sufficiently great to reduce the net loss of all components making up the fixed base of the urine, although positive balances were

not attained. In the early recovery period, the continued production of ammonia at a high rate permitted the rapid restoration of base reserves. The marked positive balance of sodium in subject P and of all components of fixed base except calcium in subject H is clearly evident in Figures 2 and 4.

Points of some possible therapeutic interest in clinical acidosis are the low plasma levels of phosphate and potassium observed during the recovery period despite the positive urinary balances. These low plasma levels undoubtedly result from the replenishment of depleted cellular reserves of potassium and phosphorus from circulating stores. Reductions in plasma levels of the magnitude observed in these experiments are inconsequential, but are in qualitative agreement with those of more significant magnitude observed following recovery from diabetic ketosis (13, 33).

The cause of the inverse relationship between chloride and bicarbonate reabsorption and the additional excretion of chloride and sodium in moderate and severe acidosis are interesting points for speculation. According to Walker *et al.* (34) the ionic pattern of the glomerular filtrate, which approximates that of the plasma, is rapidly altered during passage of the fluid through the proximal tubule. Although the sodium concentration is essentially unchanged, the chloride concentration of the residual tubular fluid rises as fluid is absorbed. One must conclude that sodium and water are reabsorbed proportionally, that chloride is absorbed infraproportionally to water, and that some other ion, most probably bicarbonate, is absorbed supraproportionally. According to Wesson *et al.* (30) the active absorption of sodium and its attendant anions in the proximal tubule is the origin of the osmotic force which causes the absorption of some 80 per cent of the filtered water in this segment. Possibly the tubule is relatively indifferent within narrow limits to the nature of the anion presented to it, *i.e.*, whether chloride or bicarbonate. Thus with moderate conversion of bicarbonate to chloride diminished absorption of the former might be more or less exactly compensated by increased absorption of the latter as a semipassive partner of sodium. However, the conversion of a significant proportion of the bicarbonate to chloride might be expected to reduce total anion absorption, for from Walker's data it

would appear that chloride absorption proceeds at a somewhat slower rate than bicarbonate absorption in the proximal segment. Reduction in total anion absorption in this segment would restrict cation (sodium) and water absorption to essentially equivalent degrees. The excess sodium over and above that normally absorbed in the distal tubule would be excreted in the urine. It is evident from Tables I and II that the magnitude of these changes is very small, a fact which renders their demonstration difficult. Nevertheless it is such small changes which underlie the negative sodium balance, diuresis and loss of weight which characterize ammonium chloride acidosis.

One is forced to the conclusion that such changes in glomerular filtration rate as occur in ammonium chloride acidosis cannot be directly responsible for changes in sodium and chloride balances. Thus qualitatively similar results in overall electrolyte balance were obtained in subjects P and H, yet in the former filtration rate fell and in the latter it rose. This fact alone, were it not supported by other evidence referred to in the first part of this paper, would justify correction of reabsorption to a standard rate of filtration for comparative purposes. This does not imply that a change in filtration rate may not modify the extent of a positive or negative balance by altering the quantity of electrolyte delivered into the distal segment of the renal tubule. Indeed there is evidence that such variations in filtration rate do alter electrolyte balance significantly (30).

That the increased excretion of ammonia and titratable acid in acidosis and for the first few days of recovery are compensations which restrict the loss of fixed base and restore the base reserves, is well recognized. Undoubtedly both serve to increase the distal tubular salvage of base, for the mechanisms for ammonia and acid excretion are both located at this site (35, 36). The former accomplishes the exchange of NH_4^+ ions for B^+ ions, the latter, the exchange of H^+ ions for B^+ ions (29). Thus in acidosis the delivery of additional base into the distal segment in consequence of diminished proximal tubular absorption is compensated by a greater exchange of H^+ and NH_4^+ ions for that base. Since as shown in Figure 6, the output of ammonia increases as rapidly as that of titratable acid following the

ingestion of ammonium chloride, it is reasonable to assign these increases to a single stimulus. Basically this stimulus appears to be a reduction in the quantity of bicarbonate delivered into the distal segment of the renal tubule, or more indirectly to a reduction in plasma bicarbonate concentration, for this latter factor largely determines the former in any given individual. According to views previously expressed there is a single transfer mechanism which exchanges H^+ ions for B^+ ions across the distal tubular epithelium (29). This mechanism is limited to some extent in transfer capacity and in the H^+ ion gradient which it can develop between blood and tubular urine. At normal plasma levels, that quantity of bicarbonate which reaches the distal tubule nearly saturates the exchange mechanism, *i.e.*, bicarbonate bound base is exchange for H^+ ion and carbonic acid is formed. The urine formed is only moderately acid and contains relatively small quantities of titratable acid, for the carbonic acid is largely dehydrated to CO_2 and water. Because of low acidity little ammonia diffuses into the urine. At reduced plasma levels such as those observed in acidosis, little bicarbonate reaches the distal tubule. Fixed acid buffers are converted to titratable acid to the limit of the capacity of the kidney to establish an high hydrogen ion gradient. Because of high acidity of the urine ammonia diffuses from its site of formation in the distal tubular cells into the tubular lumen. This latter process, by reducing the hydrogen ion gradient between blood and urine permits the continued exchange of H^+ for B^+ ions. In essence, in the absence of sufficient bicarbonate or fixed buffer in the tubular urine to provide the requisite base to saturate the exchange mechanism, that base is derived from salts of strong acid by the substitution of ammonia as the neutralizing ion.

It is obvious that some factor other than plasma bicarbonate must condition the rate of excretion of ammonia, for ammonia output increased progressively during the five-day period of acidosis, although plasma bicarbonate had reached its lowest point by the third day. The nature and mode of operation of this factor is unknown at present.

SUMMARY

Ammonium chloride was given in divided dosage to two healthy adult subjects in order (a)

to relate the rates of excretion of the several ions to their respective plasma concentrations; (b) to determine the nature and extent of the changes in glomerular filtration, renal plasma flow, and ionic reabsorption which underlie altered excretory functions in metabolic acidosis; and (c) to study the time relations of the renal compensations to acidosis.

Following the ingestion of ammonium chloride, plasma bicarbonate fell in exact proportion to the increase in plasma chloride, so that the sum of these anions remained unchanged. During the recovery phase, plasma bicarbonate rose more rapidly than chloride fell, resulting in an increase in the sum of these anions. Although the ability of the renal tubules to reabsorb chloride increased as the load of bicarbonate presented in the filtrate was reduced, the increase was not sufficient to prevent loss of chloride in the urine. Early in acidosis the excess urinary chloride was neutralized for the most part by sodium derived from body buffers. Loss of sodium resulted in a moderate reduction of plasma sodium concentration which was limited by the excretion of nearly equivalent quantities of water.

Three different mechanisms were responsible for the restriction of sodium loss and the restoration of body buffer reserves: (a) following the ingestion of an acidifying salt, the increase in chloride reabsorption per 100 ml. of glomerular filtrate significantly diminished the loss of fixed base; (b) the increased excretion of potassium and calcium on the second and third days of acidosis permitted a decrease in sodium excretion although total fixed base loss increased; (c) ammonia and titratable acid production increased progressively during acidosis permitting the continued excretion of acid with a reduction in the loss of fixed base. A lag in the return of ammonia to normal during recovery resulted in an over-compensatory increase in plasma sodium and bicarbonate.

Despite the fact that glomerular filtration rate and renal plasma flow varied in opposite directions in the two subjects during ammonium chloride acidosis, sodium, potassium and chloride balances followed a similar pattern. Chloride reabsorption increased and sodium and potassium reabsorption decreased per 100 ml. of glomerular filtrate in both subjects. Despite the diminished absorption and increased excretion, plasma potas-

sium was maintained at a nearly constant level, thus indicating the release of intracellular stores of this ion. Phosphate likewise was released from either cellular or osseous stores.

Alterations in electrolyte pattern and balance during acidosis were also studied in acute experiments. It was observed that ammonia and titratable acid excretion increased at equivalent rates and to approximately the same extent within the first two hours after the ingestion of an acidifying salt. However, only a small fraction of the maximal rate of ammonia formation is attained within this time interval. The basis for the delay in attainment of the maximal rate of ammonia excretion early in acidosis and the persistence of a high rate of excretion during recovery is unexplained at present.

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THE EFFECTS OF HISTAMINE ON RENAL FUNCTION IN HYPERTENSIVE AND NORMOTENSIVE SUBJECTS¹

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Little is known of the effects of histamine upon the renal circulation. According to Dale and Laidlaw (1), the rate of urine flow after the injection of histamine follows roughly the arterial blood pressure; their renal plethysmographic studies showed a decrease in the volume of the kidney, possibly due to active arteriolar constriction. These observations have generally been confirmed by other investigators (2-4), who found a reduced blood flow in laboratory animals and an oliguria in human beings. Following administration of histamine to human subjects, Bjering (5) noted a fall in the clearances of urea and creatinine which he ascribed to changes in renal circulation.

The favorable effect of antihistaminic drugs upon experimental nephritis in rabbits and on albuminuria and hematuria in certain cases of human glomerular nephritis suggests that histamine-like substances may cause the glomerular vasodilatation and increased permeability observed in the latter disease (6, 7). Some human subjects manifesting arterial hypertension of the "neurogenic" type (8) show a definite hypersensitivity to histamine and respond to the intracutaneous injection of a small dose with a so-called "diencephalic blush" (9). Because of these observations bearing on the influence of histamine upon renal and vascular disease, an attempt was made to investigate further the effect of histamine upon renal function. We describe below the influence of a subcutaneous injection of histamine upon renal function as measured by the clearance technique.

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SUBJECTS AND METHODS

Experiments were performed upon five patients with essential hypertension without ascertainable antecedent renal disease, and upon five normotensive patients.

Mannitol and para-aminohippurate (PAH) were measured in blood and urine during five or six consecutive periods of 10 to 20 minutes duration. After a priming dose had been administered, the blood levels of mannitol and PAH were maintained approximately constant by intravenous administration of a 0.9% sodium chloride solution containing these substances, at a rate of 4 cc. per minute. Urine was collected by catheterization, and the bladder was rinsed with 0.9% sodium chloride solution at the end of each period. The analytical procedures used were essentially those employed by Goldring and Chasis (10).^{4, 5} Among factors considered in performing the plasma and urine mannitol blanks were non-fermentable reducing substances contributed by plasma or urine, or "Factor 1," reducing substances contributed by the yeast suspension, or "Factor 2," and adsorption or destruction of mannitol by the yeast suspension, or "Factor 3." In performing the plasma blank, all three factors were corrected for, save in the case of the three subjects, L. C., I. H., and M. L., when compensation was made only for Factor 1. In performing the urine blank, Factors 2 and 3 were corrected for, save in the case of the same three subjects, when compensation was made only for Factor 2. Data on the technique employed for these blank determinations will be presented elsewhere (11).

The renal plasma clearances of mannitol and PAH were calculated using the formula "UV/P," where "U" and "P" are respectively concentration of the substance in urine and plasma, and "V" the volume of urine excreted per minute expressed in cubic centimeters (12). The value of P at the exact midpoint of each period was calculated by interpolation between the values observed during each period. The "filtration fraction" was calculated as the ratio of the mannitol clearance to the PAH clearance.

The afferent and efferent arteriolar resistances (R_A and R_E) were computed from our data according to Lam-

⁴ The mannitol and PAH solutions administered were generously contributed by Sharpe and Dohme Company; the starch-free baker's yeast used in the mannitol analyses was given by Anheuser-Busch, Inc.

⁵ Miss Ellabeth Houghton, Mrs. Mattie Heady and Mrs. Harriet Weil rendered technical assistance in performing and reporting the investigations.

TABLE I

Renal clearances of mannitol and para-aminohippurate (PAH) in five hypertensive subjects before and after histamine injection

Subject	Period	Hista- mine dose	Urine flow	Plasma levels		Urine levels		Plasma clearances		Filt. frac.*	Blood pressure†	R _A ‡	R _E §
				Manni- tol	PAH	Manni- tol	PAH	Manni- tol	PAH				
		mgm.	cc. per min.	mgm. per cc.	mgm. per cc.	mgm. per cc.	mgm. per cc.	cc. per min.	cc. per min.		mm. Hg		
I. H. ♀	1		4.58	1.23	.0346	28.2	4.02	103	531	.193	160/100	0.0659	0.0117
193 lbs.	2		5.28	1.26	.0405	23.8	3.90	100	508	.198		.0685	.0127
64 in.	3	0.3	4.50	1.18	.0431	26.0	4.14	99	431	.230	135/90	.0543	.0183
40 years	4	0.2	5.12	1.17	.0468	28.2	4.75	123	530	.232	130/85	.0387	.0149
	5		5.12	1.18	.0473	27.4	4.61	119	500	.238	140/95	.0515	.0167
	6		4.28	1.12	.0450	29.5	4.89	112	464	.242	140/100	.0578	.0180
M. L. ♀	1		2.25	1.08	.0179	38.7	4.48	80.7	563	.143	170/105	0.0743	0.0076
199 lbs.	2		2.00	1.09	.0229	39.3	5.82	72.1	508	.142		.0825	.0084
60½ in.	3	0.4	1.94	1.08	.0266	46.2	6.92	89.8	504	.178	140/90	.0542	.0109
45 years	4		1.81	1.11	.0301	47.6	7.79	78.0	468	.167	170/95	.0810	.0112
	5		2.45	1.10	.0287	40.5	6.89	90.1	588	.163	170/105	.0693	.0090
D. B. ♀	1		3.18	1.42	.0269	41.1	3.72	91.8	439	.209	175/120	0.1050	0.0159
116 lbs.	2		3.21	1.36	.0285	42.1	4.04	99.6	455	.219	175/120	.0965	.0163
64½ in.	3	0.4	2.06	1.30	.0350	52.6	5.58	83.2	328	.254	150/110	.0955	.0283
28 years	4		2.00	1.32	.0411	52.2	6.42	79.2	312	.254	150/110	.1010	.0298
	5		2.37	1.30	.0413	45.1	5.88	82.4	337	.244	165/115	.1120	.0256
F. W. ♀	1		1.70	1.54	.0347	41.3	4.67	45.7	229	.199	150/100	0.1392	0.0283
140 lbs.	2		1.61	1.50	.0358	41.1	5.46	44.4	245	.181	150/100	.1350	.0224
66½ in.	3	0.15	1.72	1.46	.0415	39.9	5.17	47.0	214	.219	150/100	.1440	.0347
28 years	4		2.51	1.45	.0471	26.5	3.70	45.7	197	.232	130/100	.1242	.0400
	5		1.65	1.45	.0514	42.2	6.48	48.0	207	.232	150/110	.1600	.0382
L. C. ♀	1		3.71	.80	.0216	25.0	2.98	116.8	512	.228	210/130	0.1090	0.0154
234 lbs.	2		3.76	.79	.0249	25.7	3.32	122.0	501	.242	210/130	.1100	.0166
63½ in.	3		3.53	.79	.0281	26.2	4.06	118.0	511	.231	210/130	.1090	.0154
32 years	4	0.4	3.51	.88	.0349	28.5	5.04	114.0	508	.226	190/120	.0937	.0151
	5	0.3	2.29	.93	.0381	39.9	7.19	97.6	432	.226	165/105	.0838	.0177
	6		2.70	.95	.0389	37.7	6.48	106.3	449	.236	165/110	.0845	.0171

* Filt. frac. = filtration fraction = $\frac{\text{mannitol clearance}}{\text{PAH clearance}}$.

† Blood pressure = arterial blood pressure.

‡ R_A = afferent arteriolar resistance in mm. of mercury per cc. renal plasma flow per minute.

§ R_E = efferent arteriolar resistance in mm. of mercury per cc. renal plasma flow per minute.

port's formulae (13). In these computations we arbitrarily assumed a hematocrit of 0.43, a plasma protein concentration of 7.0 grams per 100 cc., and a ratio of albumin to globulin of 2.2. The injection of histamine did not influence the hematocrit values in the two experiments in which the measurement was made.

In addition to the 10 clearance experiments, the renal extraction of PAH and mannitol before and after subcutaneous administration of 0.5 mgm. of histamine was determined in three subjects by catheterization of the right renal vein,⁶ using the technique devised by Cournand and Ranges (14) for the heart and developed by Warren, Brannon and Merrill (15) for the renal vein.⁷ The

⁶ The assistance of Dr. H. A. Schroeder in performing the catheterizations is gratefully acknowledged.

⁷ Experiments in which clearance measurements and renal venous catheterizations were performed simultaneously showed a definite discrepancy between the mannitol

blood pressure was measured in the arm by the auscultatory technique.

The dose of histamine was chosen so as to elicit a moderate general reaction with flushing of the face and headache. Lachrymation, palpitation, and tachycardia occurred in some patients; the blood pressure fell regularly only in hypertensive subjects. Usually 0.3 to 0.5 mgm. of histamine, calculated as the pure base, was administered, depending on body weight; in one case (F. W.) a marked reaction was produced by 0.15 mgm., and in another (L. C.) the administration of a total of 0.7 mgm. in two doses resulted in almost no reaction and no striking changes in renal clearances. The histamine

clearance as usually calculated (UV/P) and the clearance computed from the calculated renal blood flow and the observed renal extraction of mannitol (23). However, we assume that for our purposes the clearance methods permit a useful approximation of kidney function.

F. C. REUBI AND P. H. FUTCHER

TABLE II
Renal clearances of mannitol and para-aminohippurate (PAH) in five normotensive subjects before and after histamine injection *

Subject	Period	Hista- mine dose	Urine flow	Plasma levels		Urine levels		Plasma clearances		Filt. frac.	Blood pressure	RA	RE
				Manni- tol	PAH	Manni- tol	PAH	Manni- tol	PAH				
M. S. ♀ 113 lbs. 68 in. 25 years Asthenia	1	0.4	cc. per min.	mgm. per cc.	mgm. per cc.	mgm. per cc.	mgm. per cc.	cc. per min.	cc. per min.				
	2		2.48	1.32	.0249	59.8	7.77	113	774	.146	mm. Hg		
	3		2.60	1.26	.0248	58.4	7.40	120	774	.154	110/60	0.0151	0.0055
	4		2.25	1.29	.0271	56.2	7.56	98	627	.156	110/60	.0147	.0058
	5		2.65	1.28	.0279	55.3	8.09	115	767	.150	110/60	.0182	.0072
M. C. ♀ 113 lbs. 64 in. 31 years Arthritis	1	0.5	2.77	1.25	.0273	53.2	8.30	118	840	.141	110/60	.0148	.0059
	2		2.91	1.30	.0209	45.6	5.99	102	835	.122	105/60	.0136	.0041
	3		2.22	1.24	.0212	50.0	6.62	89	693	.129	118/55	.0164	.0050
	4		2.88	1.24	.0221	49.9	6.87	115	896	.128	115/50	.0153	.0039
	5		2.10	1.21	.0222	56.5	7.62	98	721	.135	110/55	.0154	.0052
J. R. ♀ 180 lbs. 62 in. 22 years Neuro- syphilis	1	0.35	2.21	1.13	.0230	58.0	7.58	113	729	.155	110/55	.0141	.0062
	2		2.51	1.32	.0296	53.7	8.20	102	696	.147	130/80	0.0332	0.0061
	3		2.47	1.34	.0351	55.4	8.72	102	614	.166	125/85	.0361	.0081
	4		2.68	1.34	.0374	50.2	8.35	100	598	.167	125/85	.0371	.0084
	5		2.06	1.34	.0381	54.5	8.96	84	485	.173	125/85	.0452	.0108
F. C. ♂ 149 lbs. 75 in. 31 years Peptic ulcer	1	0.4	2.15	0.99	.0243	50.7	9.44	109	834	.130	132/88	0.0322	0.0042
	2		2.09	1.00	.0239	39.5	8.28	82	723	.113	122/82	.0299	.0041
	3		1.54	0.92	.0254	45.9	7.37	76	446	.172	136/92	.0556	.0118
	4		1.75	0.90	.0226	57.4	10.02	112	778	.144	132/92	.0334	.0055
	5		2.00	0.89	.0248	47.5	8.80	107	710	.151	132/92	.0360	.0063
C. F. ♀ 94 lbs. 63½ in. 21 years Peptic ulcer	1	0.3	1.48†	1.29	.0428	50.2	9.56	57†	330†	.174	90/55	0.0112	0.0158
	2		2.29†	1.21	.0388	54.1	9.64	73	569†	.179	90/55	.0060	.0097
	3		1.57	1.23	.0404	56.8	9.56	74	372	.195	100/55	.0146	.0167
	4		1.73	1.22	.0426	52.7	8.45	78	343	.217	100/55	.0079	.0132
	5		1.60	1.19	.0386	57.8	9.30	78	385	.202	100/55	.0133	.0168

* See Table I footnotes for abbreviations.
† Residual urine in the bladder after the first period may account for these differences.

was administered subcutaneously as the acid phosphate in a solution containing 1 mgm. of the pure base in 1 cc. Renal clearance measurements were made for two or three control periods before injecting histamine.

RESULTS

The most constant changes in renal clearances after the injection of histamine were a decrease, as indicated by the PAH clearance (16), in the plasma flow and an associated increase in the filtration fraction. The filtration rate, as measured by the mannitol clearance (17), was not consistently altered (Tables I, II). These characteristic features are summarized in Table III. In Figure 1 are presented schematically the mean values observed in both the hypertensive and non-hypertensive groups. In the normotensive subjects,

there appeared to be no correlation between the reduction in renal plasma flow and the systemic blood pressure, the latter showing a very variable behavior. The frequent elevation in the filtration fraction in both groups of subjects indicates that active arteriolar constriction occurred. It has been generally assumed that spasm of the efferent arterioles accounts for this elevation of the filtered portion of plasma passing through the kidney (18). Whereas four out of five hypertensive individuals showed a definite rise in the filtration fraction, such an elevation was evident in only two normotensive subjects; in two others it was questionable, occurring slowly possibly because of spontaneous changes in arteriolar tone unrelated to the injection of histamine (18). In one hypertensive subject and one normotensive subject

there were no changes in filtration fraction. The average curves (Figure 1) indicate that the mean increase in filtration fraction was about the same in both series, amounting to 12% in the normotensive group and 14% in the hypertensive patients. Two normotensive individuals appeared to be very sensitive to the injected amine.

The calculated efferent arteriolar resistance was increased in four and unchanged in one hypertensive subject. It was also increased in four (in three only slightly) and unchanged in one normotensive individual. The afferent arteriolar resistance was decreased in three and unchanged in two hypertensive patients; in normotensive subjects, changes were roughly parallel to those in the efferent resistance.

As will be seen in Table IV, the renal extraction percentage of mannitol and PAH was not consistently influenced by the injection of histamine. The data on subject F. C. presented in Tables II and IV were obtained during the same experiment.

Qualitative (heat) tests on the urine of nine of the ten patients revealed no proteinuria occurring during the clearance period immediately following the injection of histamine. The tenth patient (I. H.) exhibited traces of urinary protein antecedent to the injection of histamine; the injection caused no immediate increase in proteinuria.

TABLE III

Qualitative variations in renal function and in blood pressure in normotensive and hypertensive subjects following the injection of histamine

Subject	Plasma flow	Filtration rate	Filtration fraction	Blood pressure	RA	RE
<i>Hypertensive</i>						
I. H.	d.	i.	i.	d.	d.	i.
M. L.	s.d.	i.	i.	d.	d.	i.
D. B.	d.	d.	i.	d.	u.	i.
F. W.	s.d.	s.i.	i.	d.	u.	i.
L. C.	d.	d.	u.	d.	d.	u.
<i>Normotensive</i>						
M. S.	d.	d.	u.	u.	s.i.	s.i.
M. C.	u.	u.	u.*	i.	u.	u.*
J. R.	d.	d.	u.*	u.	s.i.	s.i.
F. C.	d.	s.d.	s.i.	s.i.	i.	i.
C. F.	d.	s.d.	i.	s.i.	s.i.	s.i.

* Actually slightly increased but probably not significantly.

i. = increased. d. = decreased. s.i. = slightly increased. s.d. = slightly decreased. u. = unchanged.

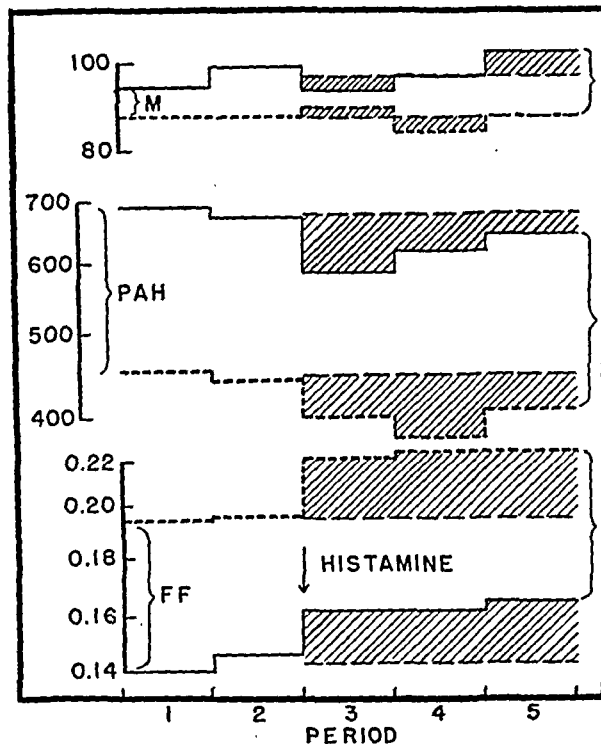


FIG. 1. SCHEMATIC PRESENTATION OF AVERAGES FOR RENAL CLEARANCES AND FILTRATION FRACTION (FF)

Broken line presents data on five hypertensive subjects (Table I); solid line on five normotensive subjects (Table II). Histamine injected at start of third period. Clearances of para-aminohippurate (PAH) and mannitol (M) expressed as cc. plasma per minute.

DISCUSSION

From these observations it would appear that the reduction in renal plasma flow following the subcutaneous administration of histamine was often due to efferent arteriolar constriction. In some cases (M. S., J. R.), in whom there was observed no change in filtration fraction, it seems likely that constriction of the afferent arteriole took place. The fall in systemic blood pressure may have played a part in reducing renal plasma flow in patient L. C.

It is known that histamine may produce vasoconstriction of arterioles, but constriction alone, wherever it might take place in the kidney, would hardly account for the absolute increase in the mannitol clearance simultaneous with a fall in systemic blood pressure observed in two hypertensive patients (I. H. and M. L.). With efferent arteriolar constriction, if relaxation of afferent arteriolar tone or increased permeability of glomeru-

TABLE IV

Renal extraction of mannitol and para-aminohippurate (PAH) in three subjects before and after injection of histamine

Subject	Time after histamine 0.5 mgm.	Para-aminohippurate				Mannitol			
		Peripheral vein level	Renal vein level	Difference	Extraction	Peripheral vein level	Renal vein level	Difference	Extraction
	<i>minutes</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>%</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>%</i>
B. C. ♂ 26 yrs. Hypertension	Before	.0314	.0095	.0219	69.8	1.19	1.05	0.14	11.7
	6	.0327	.0084	.0243	74.3	1.22	1.06	0.16	13.1
	20	.0311	.0092	.0219	70.5	1.19	1.04	0.15	12.6
	24	.0306	.0084	.0222	72.5	1.18	1.04	0.14	11.9
	42	.0288	.0078	.0210	73.0	1.15	0.95	0.20	17.4
F. C. ♂ 31 yrs. Peptic ulcer	Before	.0241	.0014	.0227	94.0	1.000	0.811	0.189	18.9
	Before	.0239	.0015	.0224	93.8	1.003	0.841	0.162	16.2
	3	.0242	.0018	.0224	92.7	0.979	0.757	0.222	22.7
	9	.0255	.0019	.0236	92.7	0.924	0.750	0.174	18.8
	17	.0249	.0022	.0227	91.3	0.909	0.778	0.131	14.4
	31	.0227	.0017	.0210	92.6	0.897	0.778	0.119	13.3
A. B. ♂ 44 yrs. Hypertension	45	.0247	.0017	.0230	93.0	0.890	0.745	0.145	16.3
	Before	.0492	.0095	.0397	80.7	1.37	1.16	0.21	15.3
	Before	.0493	.0093	.0400	81.0	1.35	1.17	0.18	13.4
	Before	.0480	.0090	.0390	81.2	1.36	1.13	0.23	16.9
	5	.0514	.0093	.0421	81.8	1.37	1.11	0.26	19.0
	21	.0534	.0105	.0429	80.2	1.42	1.15	0.27	19.0
	37	.0498	.0114	.0384	77.0	1.39	1.16	0.23	16.5

lar endothelium did not occur, one would expect little change in or a reduction of the filtration rate. The occurrence of afferent arteriolar relaxation seems to be supported by the experiment on patient I. H. presented in Table I. In the third period there was no change in the mannitol clearance, but blood flow was significantly reduced. This finding can be related to efferent arteriolar constriction. After the second injection of histamine at the start of the fourth period, however, there was a sudden increase in both PAH and mannitol clearances, the former reaching its previous level, the latter rising markedly above it. As the filtration fraction remained practically unchanged concurrent with these increases, relaxation of afferent arterioles can be considered as probably responsible for this phenomenon. Further evidence in favor of this view is found in the values for efferent and afferent arteriolar resistance; the former increased during the third period, the latter decreased during the fourth. Change in permeability of glomerular endothelium could not of itself account for the observed increase in the PAH clearance. As regards possible increased glomerular permeability, it is of interest that the slight proteinuria observed in this patient before histamine was administered did not increase.

A significant increase in the filtration rate without simultaneous reduction of renal blood flow was also evident in subject M. L. (Figure 2). Here again two interpretations are possible—efferent arteriolar spasm plus afferent relaxation, or increased endothelial permeability. The first possibility seems to be enhanced by the behavior of efferent and afferent resistance, since the former increased from 0.0084 mm. of mercury per cc. of plasma per minute to 0.0109 and the latter decreased from 0.0825 to 0.0542. It is worthy of emphasis that this absolute increase in the filtration rate did not occur in any of our normotensive subjects, suggesting that the glomerulus of the hypertensive kidney may react in a characteristic manner.

We therefore interpret our data bearing on the state of the afferent glomerular artery as indicating that in some subjects the administration of histamine produces constriction, and in others either no effect on, or relaxation of, this vessel. Relaxation was observed only within the hypertensive group of subjects.

There was no consistently pronounced effect of the injection of histamine upon the urine volume. Changes in the reabsorption of water seemed to occur spontaneously, more or less independently of the filtration rate. Data obtained from analysis

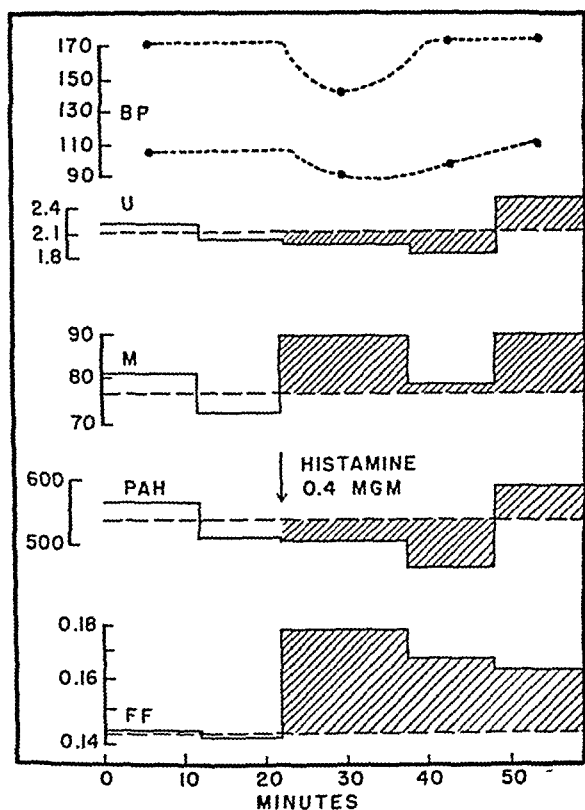


FIG. 2. EFFECT OF HISTAMINE UPON RENAL FUNCTION OF HYPERTENSIVE SUBJECT M. L.

Abbreviations and units as in Figure 1. Blood pressure (BP) expressed as mm. mercury, and urine flow (U) as cc. per minute.

of renal venous blood (Table IV) indicate that the extraction of PAH was not reduced after histamine, so that impairment of tubular function, which might explain a fall in PAH clearance, seems unlikely.

Our findings indicate that the kidney of hypertensive subjects does not appear to be on the whole much more sensitive to the action of histamine than that of normotensive subjects, at least in this small series. Moreover, individual susceptibility seems to vary widely. Noteworthy perhaps is the different behavior of the afferent arteriolar resistance in both groups. The average efferent resistance was increased from 0.0068 to 0.0088 in the normotensive and from 0.0155 to 0.0216 in the hypertensive subjects. The average afferent resistance rose after histamine from 0.0208 to 0.0249 in the normotensive but fell from 0.0995 to 0.0878 in the hypertensive group. This fall is possibly related to the fact that R_A is already very high in the latter group (19).

It is uncertain whether the observed effects upon renal physiology were due to histamine alone, or whether the action of epinephrine was involved. It is conceivable that in our experiments the administration of histamine, itself possibly inactive upon the kidney, induced a response due to adrenalin; such an effect of histamine may occur in patients with pheochromocytoma (20, 21). There is little doubt that the effects of histamine upon renal arteriolar tone are similar in some respects to those of epinephrine.

The reaction of the efferent arteriole to histamine must be emphasized, as it is elicited in this case by a so-called "hypotensive" amine. It appears that various drugs are capable of inducing an elevation in filtration fraction similar to that found persistently in hypertensive subjects.⁸

The possibility that histamine may play a part in the pathogenesis of glomerular nephritis has already been referred to. If the action of histamine is involved in the etiology of the disease, on the basis of our observations it might be expected that the filtration fraction would be found to be higher than normal. Earle *et al.* (22), however, observed an abnormally low filtration fraction in glomerular nephritis. The two patients studied by those authors who were in initial stages of their disease had manifested symptoms for three and five weeks. At this stage alterations of the capillaries may have reduced the "filtering bed." Studies performed during the very first days of the disease would provide a more acceptable basis for evaluating the possible role of histamine in its pathogenesis.

SUMMARY

1. The effect of a subcutaneous injection of histamine upon renal function was investigated in five hypertensive and five normotensive subjects

⁸ In an additional patient with hypertension studied after this work had been completed, a large dose of histamine (0.5 mgm.) produced a dramatic reaction with pallor and palpitation. The filtration fraction decreased from 0.256 to 0.100 consequent to a fall in mannitol and an elevation in PAH clearance. These findings suggested efferent arteriolar relaxation, or, as the urine flow was markedly reduced, a back diffusion of mannitol through the tubular walls. Blood pressure was not measured. Although this picture was observed only in one instance, it indicates how the effects of the same drug may vary with the susceptibility of the patient.

with the aid of measurements of the mannitol and para-aminohippurate clearances.

2. Four of five hypertensive subjects responded with an increase in the filtration fraction and efferent arteriolar resistance, probably caused by a constriction of efferent glomerular arterioles. In two of the four patients, this constriction was accompanied either by relaxation of the afferent glomerular arteriole (decreased afferent resistance), or, less likely, by increased glomerular permeability. In the fifth subject there occurred a proportional reduction of renal blood flow and filtration rate in spite of decreased afferent resistance, probably related to a fall in blood pressure.

3. In four of five normotensive patients, there were observed manifestations of efferent and afferent arteriolar constriction. One normotensive subject showed no change in renal clearance following administration of histamine.

4. Significant changes in the urine volume were not regularly observed.

5. The renal extraction of para-aminohippurate did not change after administration of histamine in three subjects.

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STUDIES OF COPROPORPHYRIN. I. THE PER DIEM EXCRETION AND ISOMER DISTRIBUTION OF COPROPORPHYRIN IN NORMAL HUMAN URINE¹

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A recent review (1) has dealt with previous studies of the urinary coproporphyrins, both as carried out in this laboratory and elsewhere. The purpose of the present series of communications is to provide newer data relating to various aspects of the coproporphyrin problem.

It is well known that two of the four coproporphyrin isomers occur in human urine (1). These naturally occurring isomers are types I and III according to H. Fischer's classification, as based upon the configuration of the four artificial aetioporphyryns (2). Apart from the difference displayed by the two isomers in ester melting point and crystal habitus, a differing physical behavior has been described as manifested in their pH fluorescence curves. This difference was first noted by Hoerbarger and Fink (3-5) who attempted to apply it to the coproporphyrin of normal urine. The crystal habitus and pH fluorescence curve of the few crystals which they obtained indicated the presence of coproporphyrin I (5). A later study by Watson (6) was likewise somewhat indecisive in that the melting point of the crystalline ester was 228° C, that of pure coproporphyrin I methyl ester being 252° C (corrected), as contrasted with the varying or dimorphic melting point of the esters of coproporphyrin III: 134°, 142°, 168° (2). The crystal habitus in this study also, was that of the type I isomer, and it was therefore concluded that in the urine employed, which was from one normal individual, the preponderant coproporphyrin was type I. At the same time it was believed that a small amount of the type III isomer was present, since a few rosettes of prisms having the type III habitus were obtained from the mother liquor. The weighed yield of crystals in

this study was 0.13 mgm., obtained from 33 liters of urine. This had seemed a fair sized undertaking, but it was dwarfed by the subsequent investigation of Grotepass (7), in which 10,000 liters of presumably normal human urine were worked up with a yield of ca. 200 mgm. of crystalline coproporphyrin ester. Grotepass' method was to adsorb the porphyrin on infusorial earth, large containers being employed into which many individuals passed their urine, the urine simply being decanted after a period of contact with the earth. The porphyrin which was isolated after subsequent purification and fractional crystallization was found to consist of about equal parts of type I and III isomers, and it was therefore concluded that the normal urinary coproporphyrin consists of approximately equal parts of the two types. Certain reservations regarding this conclusion will be discussed subsequently.

During the past nine years studies have been in progress which were designed to provide a method of isomer analysis applicable to a small volume of urine and by means of which it would be possible to make a systematic study of the urinary coproporphyrin isomer excretion without recourse to isolation of the crystalline esters. A multitude of possibilities were explored relating in the main to differential solubilities, such as that of the methyl esters in cold methyl alcohol (8) or absolute ethyl ether (9), also to differential chromatographic behavior with various solvents and adsorbents. It was previously reported (10) that pure coproporphyrin III methyl ester was readily eluted from aluminum oxide (Brockmann) by 30% aqueous acetone, while the type I ester remains adsorbed, being eluted with pure acetone. After extensive preliminary purification this method was applicable only to the porphyrin from certain urine samples. A great many alternative preliminary treatments were tried in the hope that

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one might be found which would be uniformly applicable, but none were satisfactory. During these investigations, however, a new difference in physical behavior was discovered, *i.e.*, that the red fluorescence in ultraviolet light exhibited by the type I methyl ester rapidly disappears in 30–35% aqueous acetone, in the cold, while that of the type III ester persists (11). This phenomenon has been shown to be due to a differential rate of precipitation (12). It has been made the basis of two alternative procedures for analysis of isomer distribution, A and B, which have been described in detail in separate communications (11, 12).

In the present study these procedures have been applied to a series of 24 hour urine samples from presumably normal healthy adults, in order to determine both total coproporphyrin and isomer percentage.

MATERIAL AND METHODS

Twenty-four hour urine samples were studied from 53 individuals. Thirty of these ranged in age from 19–30, ten from 30–64, and 13 from 65–86. Twenty-eight were males and 25 were females. Those between 19 and 30 in age were medical students, interns, technicians and physicians. Some of these undoubtedly had mild chemical exposure of the type encountered in a routine clinical laboratory, plus in a few instances, fumes of petroleum ether, ethyl ether, chloroform, and benzene, but in no instance was this believed to be of consequence. So far as can be determined, heavy metal exposure and alcoholism were excluded in the entire group, and there was no history of any chemical exposure in the older members of the group. The 24 hour urine samples were collected in large bottles without preservative.

Twenty-four hour urine samples were also collected from two groups of students in the Divinity School of the University of Chicago, the first a group of nine during May 1946, the second a different group of ten individuals during February 1948.² Total coproporphyrin concentration and isomer distribution were likewise determined for each of these samples.

The method employed for the quantitative determination of the total coproporphyrin and isomer percentage has been described in detail elsewhere (12). Procedure A of the two alternative procedures, was used in the first 30 instances (individuals from 19 to 30 years of age). In addition procedure B was applied to aliquots of the same 24 hour urines from 18 of these individuals. Procedure B is somewhat simpler and less liable to error than A. Repeated determinations carried out on the same

urine samples with the two procedures, have shown excellent agreement, as will be evident from data presented below.

As noted in an earlier paper (12), the fluorescence measurements have been made in a Klett fluorophotometer, using a fluorescein solution for the balanced photo cell. In this paper a typographical error unnoticed in correction of the proof, indicates that the fluorescein concentration is 3 Gm.%, whereas the correct value is a 3 mgm.%. This is an arbitrary concentration in any event which would have to be calibrated for any given instrument, against known concentrations of pure coproporphyrin.

Recently one of us (S.S.) has devised a simpler method for determination of the total urinary coproporphyrin, applicable to 5 cc. of urine. The results obtained with this procedure indicate that the true values for the UCP are approximately 20% higher than those obtained with the method (12) employed in the present study. This work will be described in a separate communication.

RESULTS

The results of the duplicate determinations with procedures A and B are given in Table I. From this it is evident that the two procedures are in good agreement.

TABLE I
Duplicate determinations with procedures A and B

No.	% type III by Procedure A	% type III by Procedure B
1	9	10
2	15	16
3	10	12
4	30	27
5	28	30
6	18	18
7	17	17
8	31	30
9	30	29
10	34	36
11	20	21
12	20	20
13	10	12
14	18	20
15	15	13
16	22	22
17	8	7
18	23	22

The data for the 53 individuals are given in Table II. As will be noted this table includes the values for the total urinary coproporphyrin (UCP) and the percentage of the two isomers. The frequency distribution of the UCP, and of each of the isomers, I and III, is shown in Figure 1.

The data for the Chicago Divinity students are given in Table III.

² We are indebted to Miss Elaine Katz of the Argonne National Laboratory, Chicago, for obtaining and sending us these samples.

TABLE II

Total UCP and isomer distribution in 53 normal subjects

No.	Sex	Age	Occupation	Total UCP	% type I	% type III
				$\gamma/24$ hrs.		
1	M	21	Med. student	80	91	9
2	M	22	Med. student	38	85	15
3	M	20	Med. student	85	90	10
4	M	23	Med. student	69	70	30
5	M	22	Med. student	57	72	28
6	M	22	Med. student	45	82	18
7	M	21	Med. student	42	83	17
8	M	21	Med. student	63	69	31
9	M	22	Med. student	74	70	30
10	M	25	Pre-Med. student	63	66	34
11	F	22	Med. student	25	80	20
12	F	21	Med. technician	46	80	20
13	F	22	Med. technician	58	83	17
14	F	28	Med. technician	63	79	21
15	F	19	Nurse	25	79	21
16	F	25	Med. technician	99	90	10
17	F	20	Med. technician	69	82	18
18	F	22	Med. technician	70	85	15
19	F	22	Med. technician	75	78	22
20	F	20	Med. technician	59	92	8
21	F	20	Med. technician	73	77	23
22	M	25	Med. student	93	65	35
23	M	23	Med. student	65	91	9
24	M	23	Med. student	17	92	8
25	M	23	Med. student	38	80	20
26	M	27	Med. student	79	87	13
27	M	27	Med. student	49	78	22
28	M	23	Med. student	52	81	19
29	F	22	Student	43	78	22
30	F	30	Nurse	28	82	18
31	F	59	Housewife	26	79	21
32	M	62	Pathologist	90	70	30
33	M	57	Pathologist	28	78	22
34	F	45	Housewife	58	80	20
35	F	52	Housewife	76	62	38
36	M	70	Businessman	67	60	40
37	F	65	Housewife	79	62	38
38	F	40	Teacher	45	84	16
39	F	38	Housewife	40	80	20
40	M	42	Businessman	50	82	18
41	F	33	Librarian	65	71	29
42	F	30	Nurse	41	68	32
43	F	82	Old people's home	56	62	38
44	M	69	Old people's home	66	48	52
45	M	86	Old people's home	37	72	28
46	F	73	Old people's home	36	68	32
47	F	75	Old people's home	25	58	42
48	M	84	Old people's home	90	63	37
49	F	78	Old people's home	36	54	46
50	M	82	Old people's home	36	66	34
51	M	79	Old people's home	62	73	27
52	M	86	Old people's home	43	59	41
53	M	82	Old people's home	28	84	16

TABLE III

Total coproporphyrin and isomer distribution in 24 hour urine samples from Divinity students

Group 1. May 1946				Group 2. Feb. 1948			
No.	Total UCP	% type I	% type III	No.	Total UCP	% type I	% type III

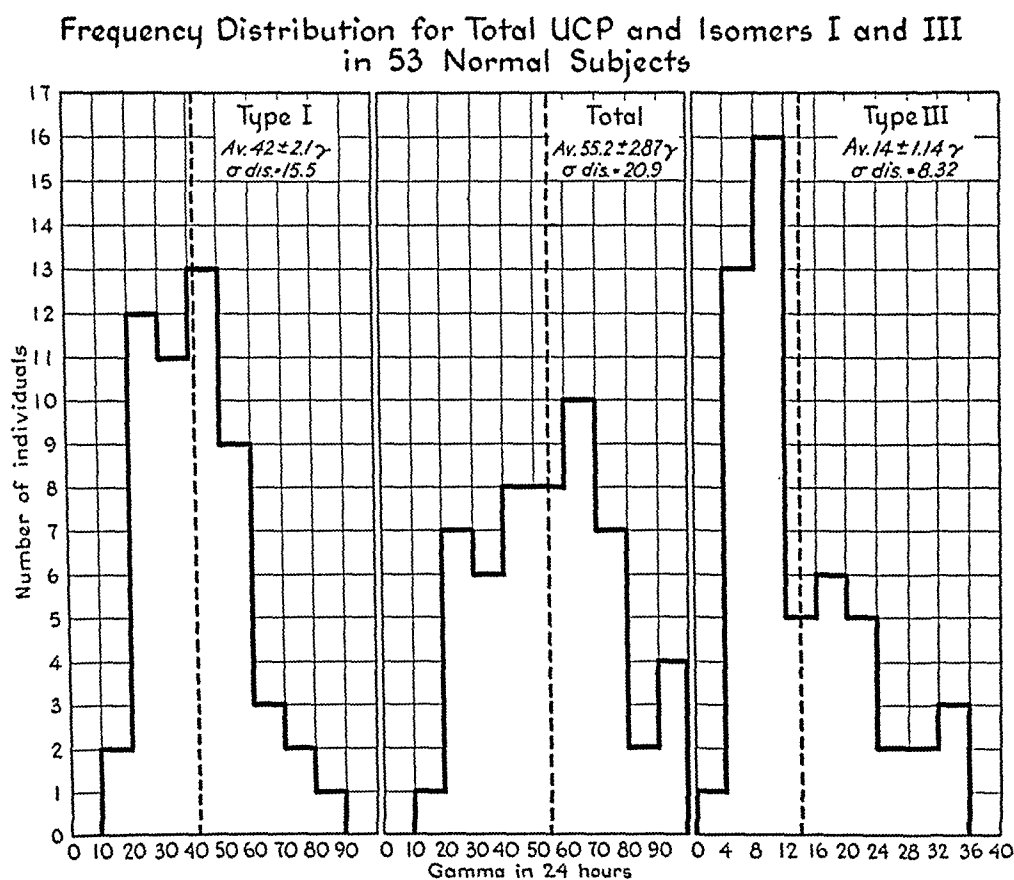


FIG. 1

or use of alcohol, but later members of the group who exhibited the same increases of the type III isomer were students in the Divinity School who had no known exposure to metals or chemicals which might produce abnormality.

In order to be doubly certain on this point, samples were collected in May 1946, from a group of nine of these students (Group 1 in Table III), and were sent to this laboratory for the isomer analysis with the results which have been given in Table III. These may be contrasted with data obtained in February 1948, from another group of 11 students at the same school. Unfortunately, it was not possible to obtain samples again from any of the individuals of the 1946 study. There is every reason to assume, however, that the habits and environment of the two groups were essentially the same. The only known difference of possible significance was the installation of a new city water filtration system for that area of Chicago, early in 1947. Thus there is the possibility that a change in water supply was responsible for the rather striking reversal in isomer-ratio noted in Table III. Other possibilities, however, are not

excluded, and further studies in other areas, both urban and rural, would be of interest with relation to such factors as water supply, air contamination and occupational or environmental chemical exposure.

In the meanwhile it is evident from the present data that a preponderance of the type I isomer is characteristic of normal adults in the Minneapolis area. It is believed that this should be regarded as the norm, since it is well established that heavy metal or chemical intoxication even though of such mild degree as to be symptomless, nevertheless causes significant increases of the type III isomer. This will be considered in a subsequent paper, in relation to the effect of alcoholism. The phenomenon of reversal of the isomer ratio within a normal total value, has been noted in certain of the "control" personnel studied by Schwartz and associates (14). The relative or even absolute decrease of type I isomer in these instances points to a closer relationship between the two isomers than hitherto entertained. Although not necessarily reciprocal, the relationship appears to be one of a balanced production normally favoring type I, but

easily upset in favor of type III by chemicals, metals and possibly other factors which will be discussed in subsequent papers.

In so far as the study of any given individual or group of cases in one area is concerned, it is recognized that before conclusions could be drawn as to the significance of reversal of the isomer ratio, it would be necessary to establish the ratio for presumably normal individuals in that area. With the method used (12), it is believed that where increases above a total of 100 γ are encountered, values of 40% or more of type III isomer may probably be regarded as abnormal even though control data for that area are not available.

In animal studies it is likewise possible that local differences will be found to prevail. Our own observations (in both Minneapolis and Chicago) have thus far indicated that the type III isomer comprises about 80% of the total in urine from healthy rabbits, and about 50% from that of healthy dogs. Whether this higher ratio of type III is due to metal or chemical contamination of the diet, as for example, from the cages, has not been determined.

SUMMARY AND CONCLUSIONS

1. The total urinary coproporphyrin (UCP) and the isomer distribution have been studied by means of the differential precipitation, or "fluorescence quenching" method, in 24 hour urine samples of 53 presumably normal adult humans of both sexes, living in Minneapolis and ranging in age from 18 to 86.

2. The UCP in this group ranged from 17 to 99 γ /24hrs., with an average of $55.2 \pm 2.87 \gamma$.

3. The percentage of type III isomer was 40 or higher in five of the 53 individuals. In three of these it was from 46 to 52%. In 48 it was less than 40% and of these, 43 were below 35%. In terms of gamma/day the type III isomer ranged from 1.3 to 34.4, with an average of $14 \pm 1.14 \gamma$.

The type I values ranged from 14.5 — 89.1 γ with an average of $42 \pm 2.1 \gamma$.

4. Twenty male Divinity students, living in the area of the University of Chicago, were also studied as to total UCP excretion and isomer distribution. Samples from the first group of nine were

obtained in May 1946. These contained from 58 to 82% of type III isomer within a normal value for total UCP. The second group of 11 was studied in February 1948; in these the range was 56 to 88% of type I isomer. The possibility is considered that a change in the water supply in 1947 may explain the return to the normal ratio.

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STUDIES OF COPROPORPHYRIN. II. AN INVESTIGATION OF THE CONTRIBUTION TO THE URINARY COPROPORPHYRIN OF HEMOGLOBIN AND OF BACTERIAL METABOLISM IN THE INTESTINAL TRACT^{1, 2}

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Stokvis (1) advanced the idea that blood in the intestinal tract was the source of the urinary porphyrin. Schumm (2) also held this belief. Subsequent studies, notably those of Fischer and Hilmer (3), Brugsch (4, 5), and Kaunitz (6) indicated that a variable fraction of the urinary coproporphyrin was thus derived. The presence of a porphyrin in meconium (2, 7, 8), later identified by Waldenström as coproporphyrin I (9), together with a number of other evidences of an endogenous formation, which are discussed in detail in recent reviews (10, 11), have made it reasonably clear that at most not more than a certain fraction of the urinary coproporphyrin (UCP) could be regarded as originating in the intestinal tract. The purpose of the present investigation was to obtain more definite information as to the size of this fraction, under various experimental and clinical conditions. It was also determined to study the relative percentage of coproporphyrin isomers composing any increases which might be observed, since previous studies bearing on these questions had dealt only with the total coproporphyrin. It was conceivable on *a priori* reasoning, that any one or a combination of the following might alter the total coproporphyrin concentration or the isomer distribution in the urine:

(1) A formation of coproporphyrin III from hemoglobin, by the bacterial flora of the colon, with reabsorption and excretion in the urine. Jakob (12) showed that pure cultures of *E. coli* produce small amounts of coproporphyrin III in the presence of hemoglobin. Snapper (13-15)

had previously shown that hemoglobin is converted in small part to protoporphyrin in the colon, and Kämmerer (16) ascribed its formation to a symbiotic activity of the bacterial flora. Hill and Holden (17) showed, however, that pure cultures of *E. coli* liberated free protoporphyrin from hemoglobin; this was confirmed by van den Bergh and co-workers (18). No direct evidence has been described, however, for the intestinal formation of coproporphyrin III from hemoglobin, although the above-mentioned work of Jakob's suggested that this might take place. In a previous study (19), it was shown that in the presence of complete biliary obstruction due to cancer, coproporphyrin could not be demonstrated in the acholic feces even when there was bleeding into the upper gastrointestinal tract. This observation does not favor an origin from hemoglobin in the bowel, but the possibility could not be excluded that coproporphyrin thus formed had been entirely reabsorbed.

(2) A formation of coproporphyrin, either I or III, as an independent bacterial synthesis in the absence of hemoglobin. Fischer and co-workers (3, 20) demonstrated that yeast cells undergoing autolysis, synthesize coproporphyrin I. Kench and Wilkinson (21) have recently shown that yeast cells elaborate both isomers, the preponderance depending on the conditions of the culture. Coulter and Stone (22) found that diphtheria bacilli in broth cultures form coproporphyrin in an amount roughly proportional to that of the toxin produced. Gray and Holt (23) have recently demonstrated that this is the type III isomer and that it is formed in relatively large amount under suitable conditions of growth. All of these observations suggested that the bacterial flora of the human colon might, at least under certain circumstances, produce either coproporphyrin I or III, and, if it were absorbed, might thus bring

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² A portion of this study was included in a thesis submitted (by E. A. L.) in partial fulfillment of the requirements for the Ph.D. degree, University of Minnesota.

about an increased urinary excretion of the corresponding isomer. von Mallinkrodt-Haupt (24) has, in fact, postulated that the urinary coproporphyrin is wholly related to the bacterial flora in the colon, and that the increases described in pernicious anemia are related to the effect of achylia in altering the flora. It will be seen subsequently that the evidence in favor of this theory is indeed very meager.

(3) The further possibility had to be considered that liver function plays an important role with respect to coproporphyrin I or III, which might originate in the intestinal tract whether from hemoglobin or bacterial metabolism. Kaunitz' study (6) suggested that coproporphyrin is absorbed from the intestine but that it does not appear in the urine in appreciable amount unless there is associated liver functional impairment. Thus, after oral administration of 20 Gm. doses of crystalline hemoglobin, significant increases of urinary coproporphyrin were observed only in cases of liver disease, or in instances where liver injury was postulated as a result of severe anemia or toxemia. Kaunitz assumed but did not prove that

the increases which he noted were composed of type III isomer, derived from the fed hemoglobin. Kaunitz (6) and Boas (25, 26) described increases of urinary coproporphyrin following gastrointestinal bleeding due to peptic ulcer or malignancy. This increase was likewise assumed to be composed of the type III isomer, although it was not isolated. There is also the possibility that some degree of liver functional impairment had occurred with resultant increase of type I rather than type III, an association which has been discussed previously (11), and in favor of which there is abundant evidence in the literature (5, 10, 27-29). It seems less probable, however, that this factor would be important after the simple ingestion of blood by normal individuals, which was reported by Schumm (2), and Fischer (3) to be followed by an increased urinary coproporphyrin of undetermined type. This was not confirmed by other investigators (14, 15, 30, 31), nor were Borst and Königsdörffer (32) able to detect the appearance of any porphyrin in the blood plasma after ingestion of coproporphyrin.

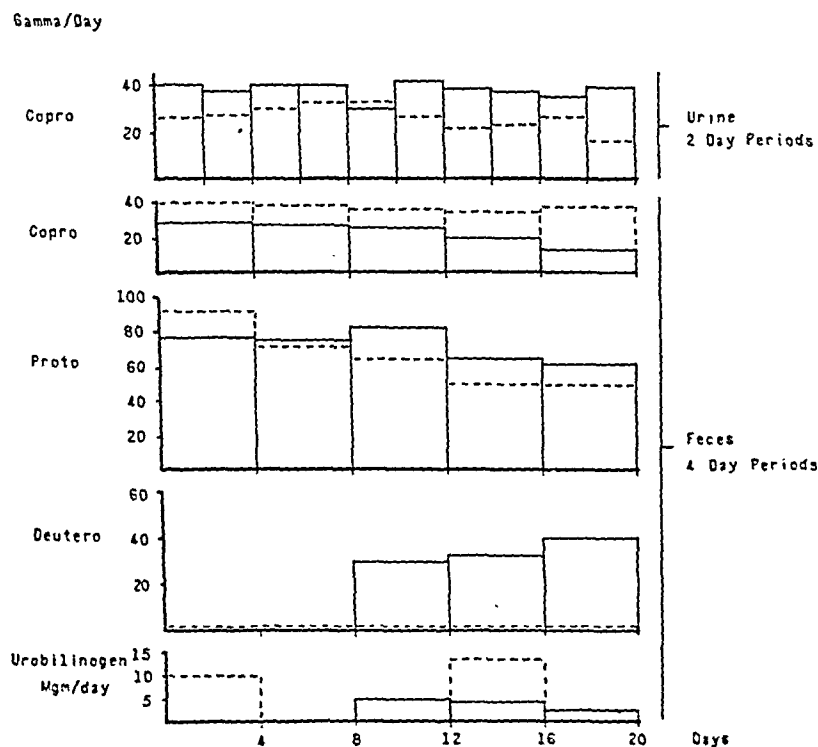


FIG. 1. NORMAL EXCRETION OF PORPHYRIN IN DOGS

Dog No. 1: Solid lines; diet—milk.

Dog No. 2: Broken lines; diet—"kibbled" dog food and milk.

MATERIAL AND METHODS

In view of the above considerations, the present study was planned as outlined in the following:

- I. Studies on dogs.
 - A. *Feeding of blood*—two dogs.
 - B. *Feeding of raw meat*—two dogs.
- II. Studies on human subjects.
 - A. *Cases having blood in the gastrointestinal tract due either to hemorrhage or to feeding of blood*:
 Hemorrhagic gastritis, one case; malnutrition, one case; hepatic gumma, one case; bacterial endocarditis, one case; cholecystitis and cholelithiasis, three cases; infectious hepatitis (recovery phase), one case; carcinoma of head of pancreas, one case; cirrhosis and chronic alcoholism, two cases; idiopathic cirrhosis, one case.
 - B. *Feeding of crystalline coproporphyrins*:
 - 1) Type III isomer—3–5 mgm. orally.
 Bacterial endocarditis, one case; cholecystitis and cholelithiasis, one case; acute alcoholism, one case; active duodenal ulcer without hemorrhage, one case; cirrhosis and chronic alcoholism, one case.
 - 2) Type I isomer—3 mgm. orally.
 Cirrhosis and chronic alcoholism, one case.

The method used for determining coproporphyrin in the urine, both total and isomer distribution, was that of Schwartz and co-workers (33). The method for the feces was that described by Watson and Layne (34). This is not an exact procedure but permits a rough approximation of the three main types of porphyrins found in the feces: 1) coproporphyrin, 2) protoporphyrin, 3) deuterio- and the pseudo-deuterioporphyrins (19). The animals used were shepherd dogs, weighing 20–30 pounds, housed in standard metabolism cages which were so constructed that the urine could be collected quantitatively under a small amount of toluene preservative. During the control period, one dog was maintained on a strict milk diet, productive of marked constipation, the other on a diet of "kibbled dog food" and milk, with which the bowel movements were normal. The urine coproporphyrin determination was carried out every two days. As mentioned previously, coproporphyrin does not deteriorate significantly in this period of time. In the human experiments determinations were made on 24 hour samples of urine. The feces porphyrins in both dogs and humans were determined on four-day collections. Urobilinogen was determined by Watson's modification (35) of Terwen's method (36). Porphyrin isolations and melting point determinations were carried out according to previously described methods (19, 37, 38, 39). The total heme pigments of the feces were determined by the method of Flink and Watson (40). The crystalline

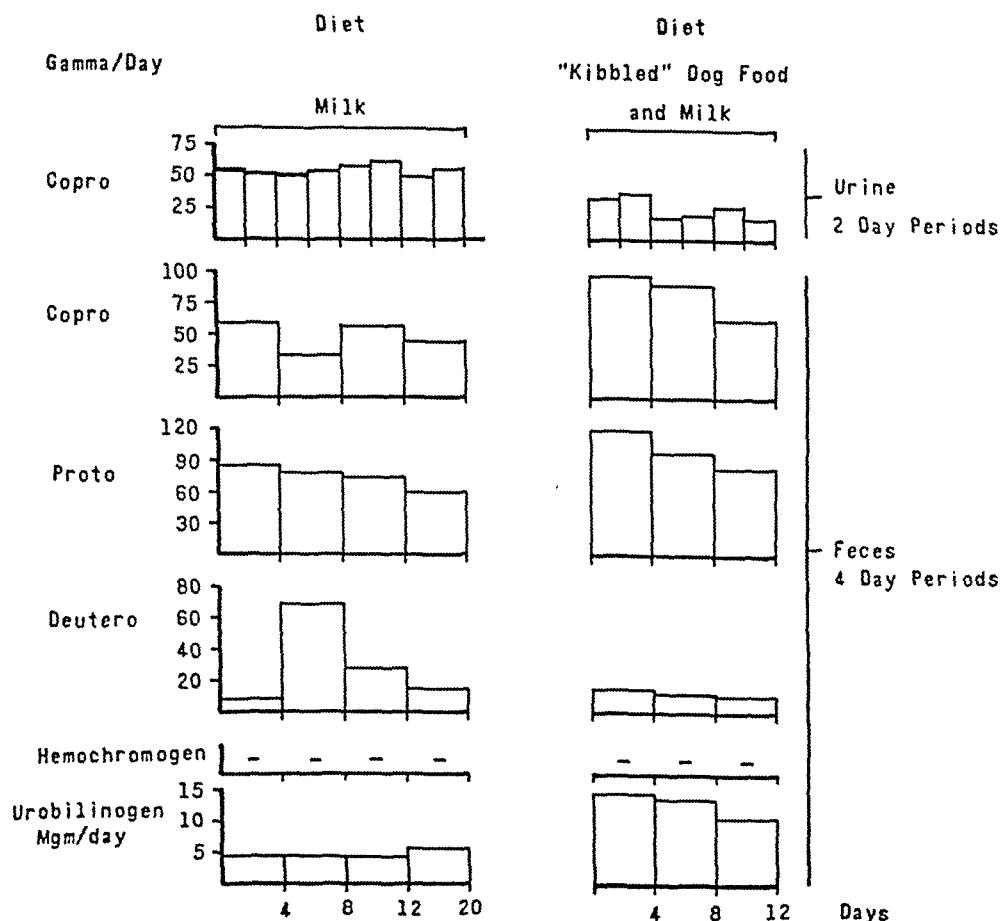


FIG. 2. EFFECT OF DIETARY CHANGE UPON THE PORPHYRIN EXCRETION
Dog No. 1.

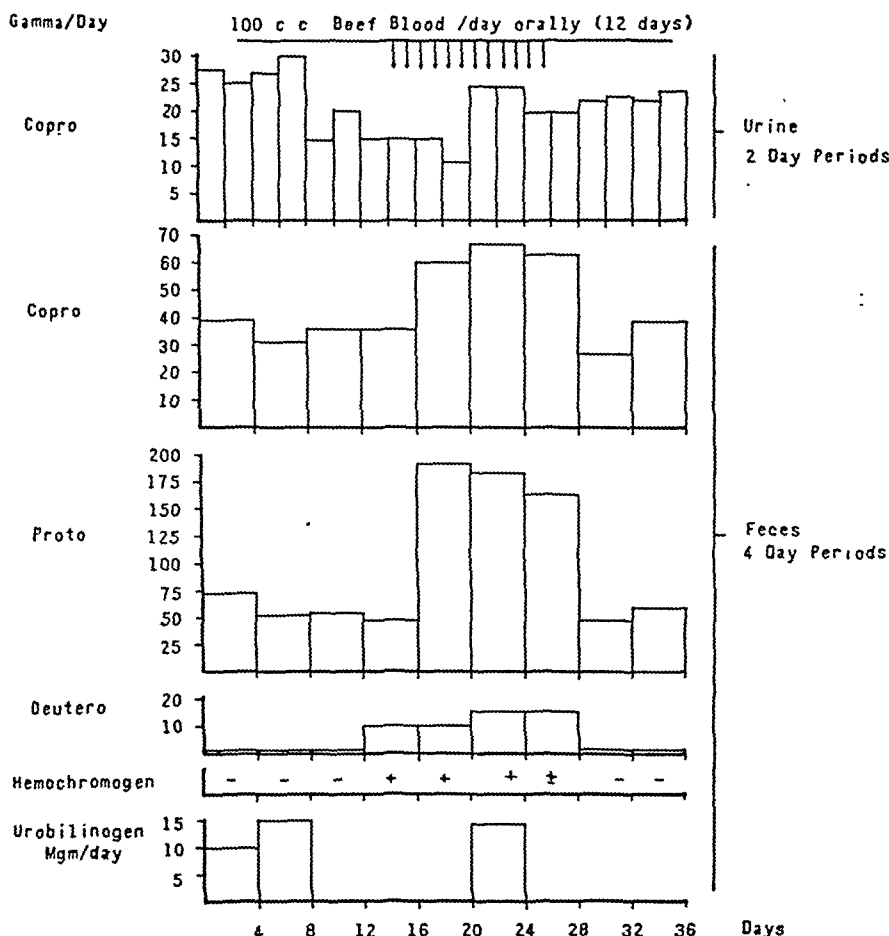


FIG. 3. EFFECT OF INGESTED BLOOD UPON THE PORPHYRIN EXCRETION IN DOGS
Dog No. 2; diet—"kibbled" dog food and milk.

coproporphyrins were fed as the hydrochlorides in a weakly acid solution. The diet was meat-free only in the cases indicated in Table I.

E. coli, *Acrobacter aerogenes*, *Pseudomonas aeruginosa*, and, in one instance, an inoculum of feces were utilized in studies of the *in vitro* synthesis of porphyrin by bacteria. The media employed were nutrient broth and tryptose broth, the same media with 5% citrated human blood added, and a synthetic medium of the Gladstone type. The periods of growth are indicated in Table II. The method of porphyrin extraction and separation in these experiments was similar to that used for the feces, save for the preliminary acetic ether extract of the liquid media. A spectrophotometric method was used in attempting to demonstrate heme pigments in plain broth (40).

RESULTS

Dog No. 1 (strict milk diet) excreted more urinary and less fecal coproporphyrin than dog No. 2 ("kibbled" dog food and milk) (Figure 1). On being changed from a milk diet to a diet of

"kibbled" dog food and milk, dog No. 1 showed a significant alteration in porphyrin excretion, *i.e.*, a decrease in urine and an increase in the feces. The total amount excreted, however, remained approximately the same (Figure 2). This interesting change will be considered subsequently. The porphyrin excretion by dogs No. 1 and No. 2 following the feeding of blood and of raw, red meat is shown in Figures 3 and 4. No significant increases in the UCP were observed after feeding blood (Figure 3).

Figure 4, which shows the effect of feeding washed, raw, red meat to dog No. 2, is also believed to be representative of experience gained in another similar study. At first glance, it might appear that the dog excreted increased amounts of urinary porphyrin following addition of meat to the diet. However, this increase was indeed slight and was maintained long after the meat was

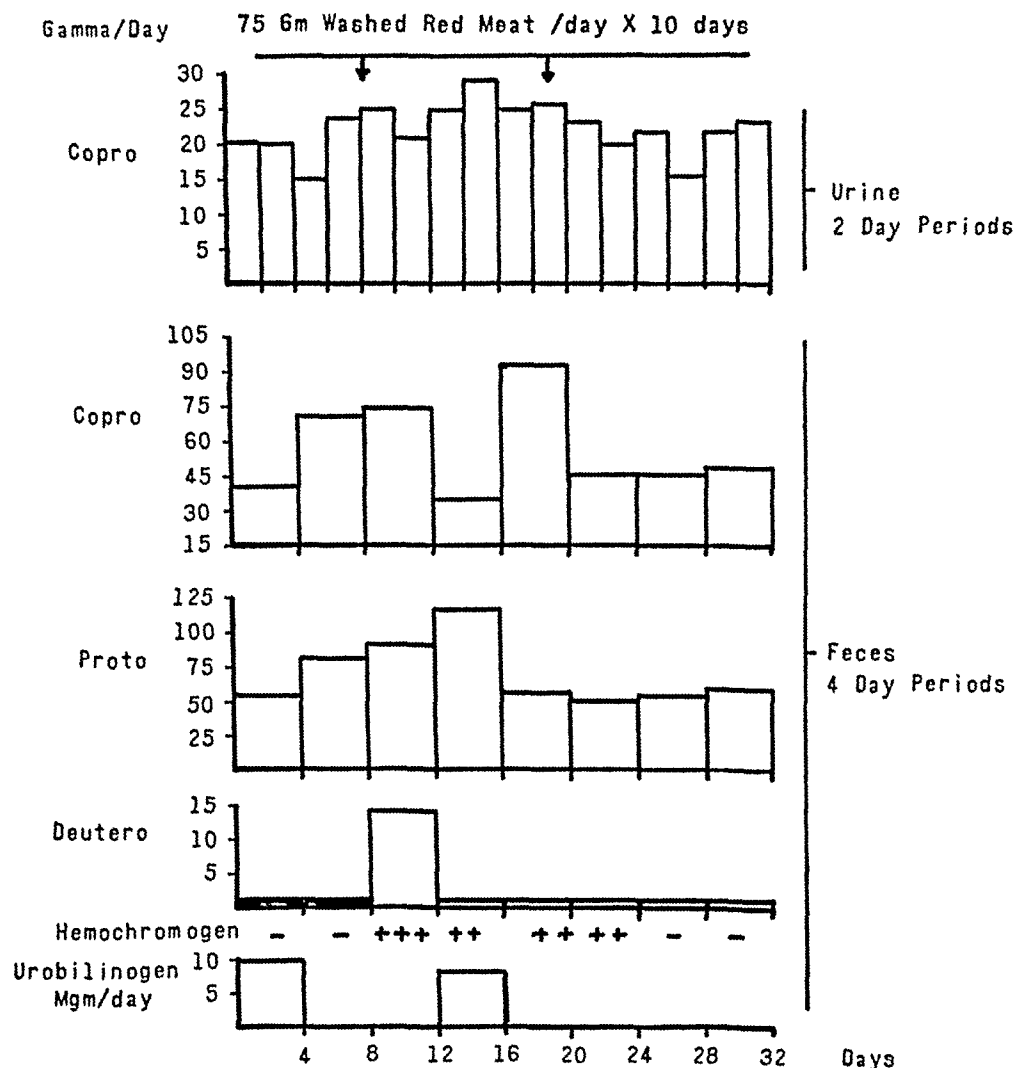


FIG. 4. EFFECT OF INGESTED RAW MEAT UPON THE PORPHYRIN EXCRETION IN DOGS
Dog No. 2; diet—"kibbled" dog food and milk.

discontinued; this may represent nothing more than a normal variation in excretion by the animal, a finding repeatedly demonstrated during the course of the dog experiments. Furthermore, the coproporphyrin methyl ester isolated from the urine during the control period and during meat feeding melted at 246°C . Since this is the type I isomer, it appears unlikely that any appreciable increase had been derived from the heme pigments of the fed meat. The findings in the feces of the two dogs following the feeding of both blood and raw meat, of which Figures 3 and 4 are representative, were quite similar. Increases in all three fractions were observed. The coproporphyrin of the feces after meat feeding was identified as the type I isomer in dog No. 2. The presence of small amounts of coproporphyrin III was strongly suggested by a highly colored mother

liquor following crystallization of the type I isomer, but further crystallization could not be effected.

Figures 5, 6, and 7 show the results of studies on several human subjects following the feeding of hemoglobin, coproporphyrin III, and in one instance, coproporphyrin I (Figure 5). Except where otherwise indicated, the studies were conducted in consecutive four-day periods. The results of studies on a 59 year old patient having outspoken evidence of cirrhosis and severe hepatic functional impairment associated with chronic alcoholism and dietary deficiency, are shown in Figure 5. Although the average excretion of urinary porphyrin is slightly greater after the feeding of hemoglobin, the occurrence of gross variations during the control period and in previous untabulated determinations, minimizes the significance of this increase. Moreover, the unexplained decrease

in the next four-day period, following the feeding of 5 mgm. of coproporphyrin III, is probably a further coincidental variation in excretion. The low values after 3 mgm. of coproporphyrin I were obtained during a later period, when the average urinary excretion was appreciably lower than that observed early in the patient's hospital course. Of principal importance is the fact that during both feeding experiments, approximately all of the fed coproporphyrin appeared in the feces and there was no increase in the urine. In one instance after the feeding of coproporphyrin III, the same isomer was easily crystallized and identified from the feces. Figure 6 shows the results of studies on a 62 year old woman with a high-grade biliary obstruction due to an impacted common duct stone. An increase in the urinary porphyrin excretion was observed on the third day following hemoglobin feeding, but during the corresponding four-day period, there was a diminished fecal coproporphyrin excretion, suggesting an increased ob-

struction to bile flow. There was no increase in urinary porphyrin excretion following the feeding of coproporphyrin III, most of which was accounted for in the feces. Further data on jaundiced subjects are shown in Figure 7: Case 12 (cirrhosis), Case 13 (calculus obstruction), and Case 14 (carcinomatous obstruction). The results of additional case studies are listed in Table I.

Case 2 (Table I) requires further explanation. This individual had taken alcohol rather continuously until the hour of admission in spite of epigastric pain and moderate hematemesis. The diet had been deficient for a matter of months prior to this time. The results of the study appear to indicate that significantly larger amounts of coproporphyrin were excreted in the urine while blood was present in the bowel. However, the "control" study, following cessation of bleeding, was conducted eight days later, when the patient was in a far better nutritional state and had not been drink-

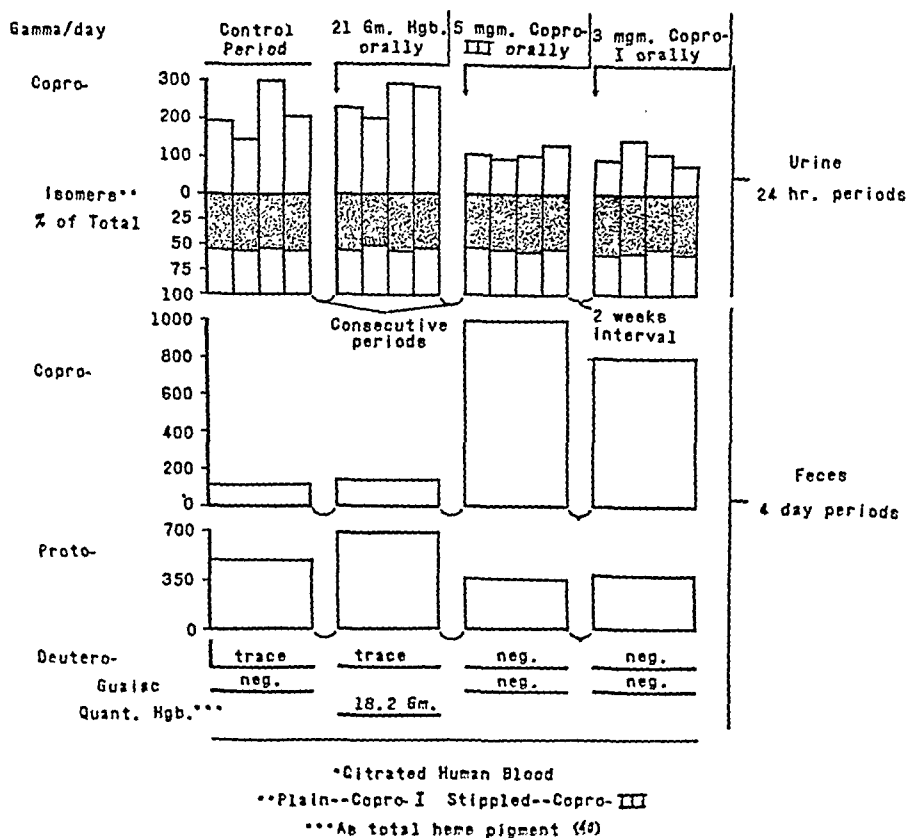


FIG. 5. EFFECT OF THE INGESTION OF HEMOGLOBIN AND OF COPROPORPHYRIN III AND I UPON THE PORPHYRIN EXCRETION IN THE PRESENCE OF SEVERE LIVER DAMAGE

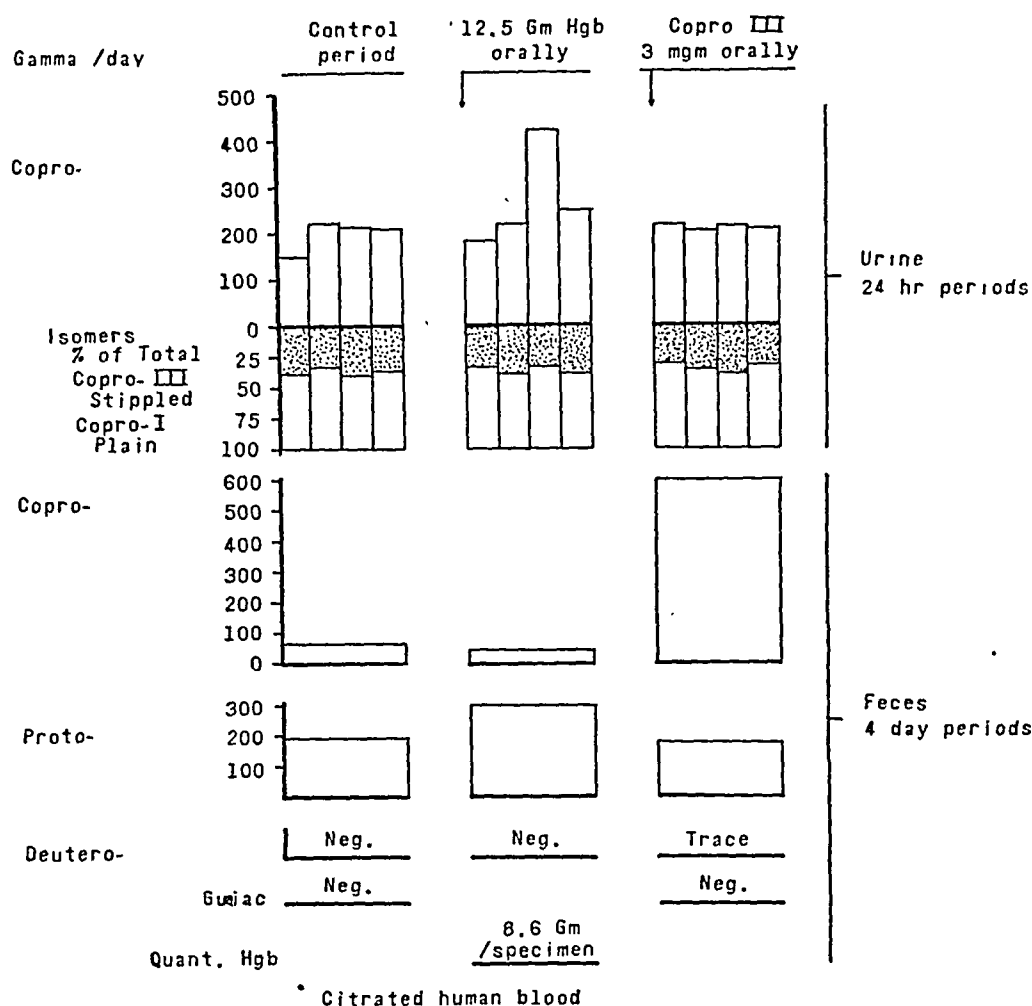


FIG. 6. EFFECT OF ORAL HEMOGLOBIN AND COPROPORPHYRIN III UPON THE PORPHYRIN EXCRETION IN HUMANS

ing. The effect of acute alcoholism upon the urinary porphyrin excretion is shown in Case 7, Table I, and will be discussed in detail in a subsequent paper of this series.

The results of studies of the synthesis of porphyrins by bacteria are shown in Table II.

DISCUSSION

The results of the studies on dogs maintained on different diets appear to permit either of two interpretations: (1) That the constipating effect of a milk diet promotes absorption of coproporphyrin from the bowel and consequent increase in urinary excretion; or (2) That the excretory function of the liver is relatively sluggish on a milk diet with the result that a larger fraction of the total appears in the urine. Since it would appear that a normal liver might be expected to handle the increment of coproporphyrin which an enterohepatic circulation due to constipation might conceivably

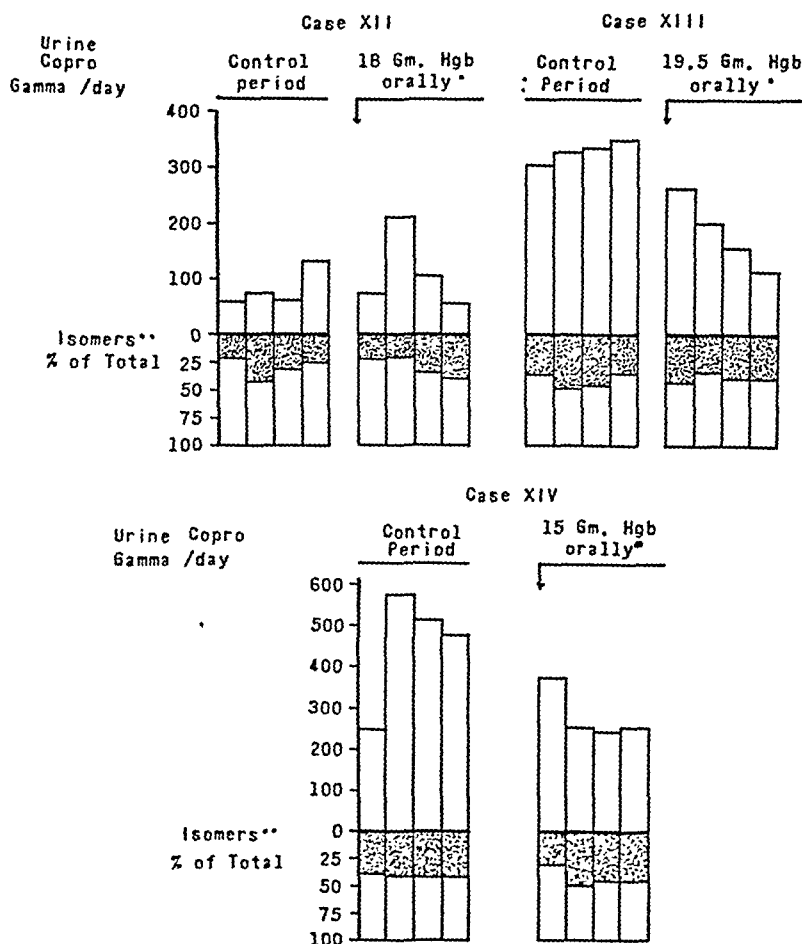
provide, we are inclined to favor the second of these two interpretations. It is quite possible that the bile flow is a major factor in the difference between the two diets; there is reason to believe that less bile would be formed on the milk diet alone because of its relatively higher carbohydrate, and lower protein content (3.5% as against 22.5%), and hence that less coproporphyrin might be excreted by the liver.

No conclusive evidence was obtained in the dogs of a process of reabsorption and increased excretion of coproporphyrin in the urine following the feeding of blood or meat. The increase of fecal coproporphyrin excretion observed in each animal in these experiments is probably related to an increased flow of bile, although a bacterial synthesis cannot be excluded. The finding that this increase is represented by the type I rather than the type III isomer favors the former rather than the latter possibility, since the coproporphyrin of

the bile has been shown to be type I, while the pre-existing evidence and that furnished by the present study indicate that bacteria elaborate type III.

The more detailed studies in human subjects also fail to furnish any conclusive evidence for a reabsorption of porphyrin from the bowel following the feeding of blood, regardless of the state of liver function. Moreover, the excretion of urinary porphyrin after the feeding of coproporphyrin III in five instances and of the type I isomer in one instance actually provides significant evidence to the contrary. Although these findings are at variance with those of Kaunitz and others after the feeding of hemoglobin or blood (3, 6) and coproporphyrin I (3), they are strongly supported in the present

investigation by the observation that the relative percentages of isomer types did not change. This finding practically excludes any appreciable contribution to the UCP of fed protoporphyrin type III (hemoglobin) or coproporphyrin III and I. Any increase in urinary coproporphyrin excretion following the feeding of either porphyrin would necessarily be of the same type. This assumption is based on chemical evidence that a transformation of one type of isomer to another would entail a disruption and complete resynthesis of the molecule. Although quantitative recoveries were seldom attained, it was obvious that the bulk of the fed porphyrin could be found in the following four-day collection of feces.



*Citratd Human Blood
 **Plain-Copro I. Stippled-Copro III
 Consecutive 24 hr. urine collections in each case.

FIG. 7. EFFECT OF THE INGESTION OF HEMOGLOBIN UPON THE URINARY PORPHYRIN EXCRETION IN THE PRESENCE OF SEVERE LIVER DAMAGE (CASE 12) AND OF OBSTRUCTIVE JAUNDICE (CASES 13 AND 14)

TABLE I
Observations on the urinary and fecal porphyrin excreted after gastrointestinal hemorrhage and the separate feeding of either hemoglobin or coproporphyrin III

Case No.	Age and Sex	Diagnosis	Date	Hgb. fed	Total heme pigment recovered in feces	Urinary coproporphyrin excretion (gamma/day)						Feces porphyrin excretion						
						Control period		After hemorrhage Hgb. feeding		After copro-III feeding		Coproporphyrin		Protoporphyrin				
						Quant. excreted	% type III isomer	Quant. excreted	% type III isomer	Quant. excreted	% type III isomer	Control period	After hem. or hgb. feed.	After copro-III feed.	Total mgm. recovered in 4 days after feed. 3 mgm.	Control period	After hem. or hgb. feed.	After copro-III feed.
1	65 ♀	Malnutrition	1946			135	28					gamma/day	95		gamma/day			
			4-12			125	30					80						
			4-13			100	25											
			4-15	20	19.8			135	24									
			4-17					168	23									
2	49 ♂	Chronic alcoholism; hemorrhagic gastritis†	4-18			100	26											
			4-19			98	25											
			5-18			120†	80											
			5-19			200	85											
			5-20			120	78											
3	39 ♀	Hepar lobatum	5-10		7.2	250	75											
			5-13			265	75											
			7-3			38	28											
			7-5			52	28											
			7-6	15		125	30											
4	62 ♂	Cholecystitis and cholelithiasis (no jaundice)*	7-7			170	30											
			7-8			45	28											
			7-9			80	29											
			7-10		12.6	130	30											
			7-3			20	26											
5	33 ♀	Subacute bacterial* endocarditis	7-6	15														
			7-7			17	26											
			7-8			60	27											
			7-9			65	28											
			7-10		14.1	75	26											
5	33 ♀	Subacute bacterial* endocarditis	6-12			65	25											
			6-14			40	25											
			6-15			45	23											
			6-16	10	8.2			65	30									
			6-17			45	28											
5	33 ♀	Subacute bacterial* endocarditis	6-18			50	25											
			6-19			48	23											
			6-25					60	30									
			6-26					120										
			6-27					100										
5	33 ♀	Subacute bacterial* endocarditis	6-27															
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5	33 ♀	Subacute bacterial* endocarditis	6-27															
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TABLE 1—Continued

Case No.	Age and Sex	Diagnosis	Date	Hgb. fed	Total heme pigment recovered in feces	Urinary coproporphyrin excretion (gamma/day)						Feces porphyrin excretion					
						Control period	After hemorrhage Hgb. feeding		After copro-III feeding			Coproporphyrin			Protoporphyrin		
							Quant. excreted	% type III isomer	Quant. excreted	% type III isomer		Control period	After hem. or hgb. feed.	After copro-III feed.	Total mgm. recovered in 4 days after feed. 3 mgm.	Control period	After hem. or hgb. feed.
7	34 ♂	Acute* alcoholism	1946 5-23 5-25 5-26 5-27 5-28 5-30		grams 115 175 85	58 44 75			105 100 105	55 50 54		95	γ/day	820§	3.28	225	γ/day
8	57 ♂	Duodenal ulcer (without† hemorrhage)	5-30 5-31 6-1 6-2 6-4 6-5 6-6			25 22 25 21	55 60 135 120		75 35 50	18 21 22		115			1.5	230	
10	31 ♀	Infectious hepatitis (recovery phase)	9-18 9-19 9-20 9-21 9-22 9-23 9-24 9-25	18.7	16.2	26 22 25 22	80 78 100 120		140 35 60 65	26 21 25 25		45				90	85
11	26 ♀	Idiopathic hepatic cirrhosis	9-21 9-22 9-23 9-24 9-25 9-26 9-27 9-28	20	17.2	35 20 37 25	160 130 75 180		65 75 95 90	30 35 34 34		180	225			325	710

Urine determinations on 24 hour collections. Feces determinations on 4 day collections. Deuteroporphyrin either lacking or present only in traces in each instance.

* Meat free diet.

† Milk and cream diet.

‡ Interval of 8 days after hemorrhagic period; feces guaiac negative.

§ Type III isomer isolated with ease.

TABLE II
Observations on the bacterial synthesis of porphyrins

Organism	Culture medium	Days cultured	Type porphyrin	Amount in gamma	Isomers
<i>B. coli</i> †	Plain Broth	15	Copro-Proto-Deutero-	35/500 cc. Trace Neg.	I = 15% III = 85%
	Blood Broth	15	Copro-Proto-Deutero-	15/500 cc.* Trace Neg.	I = 9% III = 91%
<i>Aerobacter aerogenes</i> †	Plain Broth	14	Copro-Proto-Deutero-	38/500 cc. 7/500 cc. Neg.	I = 14% III = 86%
	Blood Broth	14	Copro-Proto-Deutero-	34/500 cc. 16/500 cc. Neg.	I = 11% III = 89%
<i>Pseudomonas aeruginosa</i> †	Tryptose Broth	12	Copro-Proto-Deutero-	31/100 cc. 28/100 cc. Neg.	I = 27% III = 73%
	Tryptose Blood Broth	12	Copro-Proto-Deutero-	42/100 cc. 38/100 cc. Neg.	I = 17% III = 83%
Feces	Synthetic	11	None	None/500 cc.	
<i>Pseudomonas aeruginosa</i> †	Synthetic	11	None	None/500 cc.	
<i>Pseudomonas aeruginosa</i> †	Synthetic	11	None	None/500 cc.	

Heme compounds not demonstrable in 200 cc. of plain broth after extraction and eight-fold concentration (40).

* An estimated half of the total porphyrin lost during extraction.

† Inoculated organism recovered in pure culture at end of experiment.

The increases in UCP observed in the cases of alcoholism included in the present study, are consistent with the usual finding in acute alcoholism of distinct increases of coproporphyrin III, sufficient as a rule to elevate the UCP value into the range of 120–500 γ /day. This will be considered in detail in a subsequent paper of this series. The regular increases in alcoholics not having blood in the intestinal tract, obviate the need of ascribing to such a source the increase observed in the cases of hemorrhagic (alcoholic) gastritis included in the present study.

Previous investigators (3, 6) have utilized a diet low in protoporphyrin content as a prerequisite to the demonstration of reabsorption of porphyrin from the bowel after the feeding of blood or porphyrin. Wherever it was feasible clinically, this plan was followed in the present investigation, in spite of which, the results in the studies on humans do not indicate any differences in urinary porphyrin excretion which might be attributed to the diet.

Just as in the dog experiments, increases of fecal coproporphyrin excretion were almost in-

variably observed following the feeding of blood to humans. One notable exception occurred in the case of high-grade biliary obstruction (Figure 6) which suggests that the elevations in the other instances were on the basis of increased flow of bile into the intestine. However, an alteration of the bacterial flora and consequently of bacterial synthesis of porphyrin, due to the exclusion of bile from the intestine, cannot be excluded. In this connection, it is of interest that preliminary studies have failed to reveal any effect of aureomycin, given orally, on the UCP. It is known that aureomycin markedly inhibits the bacterial flora of the colon, especially *E. coli*.

In the studies on bacteria, pure cultures of several organisms in broth media were found capable of synthesizing both coproporphyrin and protoporphyrin. In contrast to the results of previous studies (17, 18), relatively little protoporphyrin was recovered from the culture of *E. coli* in a medium containing blood. The explanation may lie in the much larger amount of hemoglobin which was exposed to bacterial action in the previous studies; it is also possible that the longer period of culture in the present study resulted in degradation and disappearance of an undetermined fraction of the protoporphyrin. The synthesis progressed despite the absence of demonstrable heme compounds, lending support to recent tracer studies which indicate that this process proceeds from simple precursor molecules such as acetate and glycine (41, 42).

SUMMARY AND CONCLUSIONS

1. Studies in dogs have shown that a milk diet is associated with a higher urinary and lower fecal coproporphyrin excretion than a diet composed of milk and prepared (kibbled) dog food. The total amount of coproporphyrin excreted is approximately the same, and the interpretation is favored that the hepatic coproporphyrin excretory function or the bile flow, or both, are relatively diminished on a milk diet.

2. No evidence was obtained either in dogs or humans, with normal or impaired liver function, of an absorption of coproporphyrin from the intestine and excretion in the urine, following the feeding of blood or raw meat, or spontaneous gastrointestinal hemorrhage. Increases were commonly encountered in the feces in such experi-

ments, but in instances where the porphyrin was identified it was shown to be the type I rather than the type III isomer.

3. No evidence was obtained in human experiments for the absorption from the intestine and excretion in the urine of fed coproporphyrin I or III. This was true in individuals with markedly impaired as well as normal liver function. The bulk of the fed porphyrin was recovered in the feces. Any small fractions that may have been absorbed were either metabolized or reexcreted in the bile.

4. A variety of bacterial strains, in pure culture, were found to synthesize both protoporphyrin 9 (type III) and coproporphyrin III. This synthesis did not proceed on artificial media of the Gladstone type, but required substances, presumably of protein character or origin, present in broth. The synthesis of porphyrins proceeded in broth media which did not contain demonstrable hemoglobin or heme pigments.

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STUDIES OF COPROPORPHYRIN. III. IDIOPATHIC COPROPORPHYRINURIA; A HITHERTO UNRECOGNIZED FORM CHARACTERIZED BY LACK OF SYMPTOMS IN SPITE OF THE EXCRETION OF LARGE AMOUNTS OF COPROPORPHYRIN¹

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It has been generally assumed that the formation and excretion of large amounts of coproporphyrin is regularly associated with the presence of symptoms, usually of either abdominal or nervous type. Certain students of the porphyrins, notably Carrié (1) have ascribed to coproporphyrin a primary rôle in the symptoms of lead poisoning and other conditions associated with a considerable increase of coproporphyrin excretion. Thus, the abdominal pain and peripheral neuritis are designated by Carrié as "porphyrinopathic" manifestations. Intermittent acute porphyria, characterized by production and excretion of the wholly abnormal Waldenström uro-type porphyrin, is often entirely latent and symptomless for long periods, probably, even, for the individual's entire life span in some instances. So far as can be determined, however, no case has been recorded in which there was a massive and persistent, and at the same time entirely symptomless idiopathic coproporphyrinuria. At least until further knowledge may indicate a different terminology, it is believed best to use this designation, rather than porphyria, for the cases which will be described. In agreement with Waldenström, we prefer to reserve the latter term for cases in which uroporphyrin, or porphyrins of uroporphyrin type, such as the Waldenström or 260 porphyrin (2-4), are demonstrated. The term porphyria, in other words, connotes a metabolic error in which an entirely abnormal porphyrin is formed and excreted, while the term coproporphyrinuria is non-committal in the sense that while it is both physio-

logical and pathological, secondary to a variety of disturbances, the possibility exists that occasional cases, such as those described in the following, are likewise "inborn errors of metabolism."

CASE REPORTS

Case 1. W. V. Q., ♂, 26, white, chemist. This individual had no complaints and considered himself to be in excellent health.

As a graduate student he had worked for one and a half years in a laboratory in which 1-2 pounds of mercury were always kept in an open vessel. He recalls "one or two" mercury spills in the course of his own work, in which he wiped up the mercury with copper powder. Once, during this same period, about two years prior to the present study, he was pipetting mercury and some entered his mouth, but he is certain that none of it was swallowed. Also about two years ago while pipetting saturated copper sulphate solution, some got into his mouth. He thought none was swallowed but he noted a "funny" feeling in the stomach for several hours. In addition to these episodes he has been exposed only to the usual organic and inorganic compounds found in most chemical laboratories. So far as could be determined alcoholic beverages were but rarely used and only in small amount.

As a child he had had measles, mumps and chicken pox, and pneumonia at 11. His mother died of tuberculosis at 56, his father is living and well, as are three siblings. Neither the patient nor members of his family have ever noted red urine, and none have had suggestive complaints such as abdominal pain, severe constipation, or pain in the extremities. There has been nothing suggestive of light sensitivity in the patient or his relatives.

The physical, including the neurological examination was entirely normal. The blood pressure was 120/70. The routine examinations of the blood and urine were normal. There was no anemia. The blood smears revealed nothing abnormal; there was no increase in basophilic stippling. The sternal bone marrow was normal. The feces urobilinogen for three separate four-day periods was 83, 86, and 118 mgm./day; thus there was no evidence of an increase in rate of blood destruction, in fact,

¹ Aided by grants from the John and Mary R. Markle Foundation, the Rockefeller Foundation, and the Medical Research Fund of the Graduate School, University of Minnesota.

the values are rather low. There was no evidence of liver functional impairment. Thus the urine urobilinogen on three occasions was well within normal limits, 0.05, 0.06, and 0.12 mgm./100 cc. The cephalin-cholesterol flocculation test was negative. Repeated serum bilirubin determinations were well within normal limits. The urine coproporphyrin was greatly increased as noted in Table I.

TABLE I

Urine coproporphyrin—case W. V. Q. and siblings

Case W. V. Q.	Date	Concentration in γ /100 cc.
	4-24-45	218.0
	5-1-45	142.0
	5-22-45	96.5
	6-8-45	126.3
	6-16-45	255.0
	7-20-45	108.0
	9-18-45	381.5
	12-18-45	201.0
	1-23-46	449.0
Sister M.	7-11-45	2.9
	7-28-45	4.6
Brother Ro.	7-11-45	3.3
Brother Ru.	9-20-45	4.1

The urine was examined repeatedly for the presence of ether insoluble porphyrins, such as uroporphyrin or the Waldenström porphyrin, but none were demonstrable. The test for porphobilinogen (5) was made on a number of occasions and with one exception was negative. The exception was a very faintly positive reaction of doubtful significance.

The isomer distribution of the UCP was determined on two occasions with the following result:

11-16-45	86% type III
	{ 91% type III
12-28-45	{ 95% type III

The first two of the above results were obtained with the B modification of the isomer analysis method, the third with the A procedure (6a). As noted in paper I of this series (7), the results of these two techniques are essentially the same.

A spectrographic metal analysis² of the urine of W. V. Q. did not reveal any abnormal amounts of metals.

² Carried out by the spectrochemical division of the Metallurgy Laboratory University of Chicago, to whom we are indebted for the report.

TABLE II

Fecal porphyrin concentration in case W. V. Q.

Date	Coproporphyrin γ /100 Gm.	Protoporphyrin γ /100 Gm.*	Deuteroporphyrin γ /100 Gm.*
6-8-45	69000	1263	—
9-21-45	32375	381	638

* The values for proto- and deuteroporphyrins are given in coproporphyrin equivalents.

The fecal porphyrins were quantitated on two occasions with results as given in Table II.

On several occasions the isomer distribution in the urine was determined by means of the differential precipitation method (6a). From 86-95% coproporphyrin III was found. Coproporphyrin III methyl ester was repeatedly isolated in considerable amount from both feces and urine. The crystal habitus and dimorphic melting point were characteristic (8). The methods of isolation have been described elsewhere (4, 8, 9).

Gastric and duodenal contents were examined, with the results shown in Table III.

The erythrocyte protoporphyrin was determined on two occasions by the method of Grinstein and Watson (10).

9-29-45	46 γ /100 cc. of cells
3-24-46	37 γ /100 cc. of cells

On neither occasion was there any demonstrable coproporphyrin in either erythrocytes or plasma.

Examination of the bone marrow revealed a normal morphology. The protoporphyrin concentration was 37 γ /100 cc. No coproporphyrin was demonstrable.

Case 2. B. C., ♂, 31, negro laborer. Admitted to the Minneapolis General Hospital on January 23, 1948, with a diagnosis of diphtheria. A typical membrane was observed in the pharynx and throat cultures were positive for *B. diphtheriae*. The illness was not severe and there were no recognizable complications. 40,000 units of anti-toxin were given on the day of admission. The patient made an uneventful recovery. The highest temperature recorded was 102° F., on the day of admission. The temperature was consistently normal after the second hospital day.

TABLE III

Coproporphyrin concentration in gastric and duodenal contents

Time	Source	Color	Fluorescence	Coproporphyrin γ /100 cc.	Bilirubin mg./100 cc.
11:15 a.m.	Gastric	Nearly colorless	Bluish	0.6	0
11:25 a.m.	Gastric	Light brown	Medium red orange	24.0	trace
20 cc.	11:35 a.m.	Duodenal	Brown	308.0	1.5
35% MgSO ₄	11:45 a.m.	Duodenal	Golden brown	720.0	4.4
	11:55 a.m.	Duodenal	Golden brown	1327.0*	6.7

* 95% copro-III according to differential precipitation technique (6a).

The urinary coproporphyrin was studied in this case because of the previous observations that diphtheria bacilli in broth cultures produce coproporphyrin in considerable amount (11). This has recently been shown to be the type III isomer (12). The present case was one of a group of diphtheria cases under investigation with respect to possible increases in UCP. It was surprising to find that this individual was excreting unusually large amounts of coproporphyrin, and that this greatly heightened excretion persisted long after the diphtheria had disappeared. There were no symptoms other than those related to the throat, of transitory nature. There was no history of exposure to chemicals or heavy metals, and the patient denied the use of alcoholic beverages in any form. There was no evidence of liver functional impairment. Thus the serum bilirubin was: 0.2 mgm./100 cc. (1' or prompt direct) and 0.9 mgm./100 cc. total; the cephalin cholesterol test was negative and the thymol turbidity 1.9 units; the serum alkaline phosphatase was 4.0 Bodansky units.

The patient was uncooperative and, although attempts were made to keep him under observation indefinitely, he left Minneapolis toward the end of April for an unknown destination.

The findings in this case were as follows:

TABLE IV
Urinary porphyrin excretion

Date	UCP	% type III isomer	M.P. of cryst. methyl ester	Porphobilinogen reaction (5)	Uroporphyrin
	$\gamma/24$ hrs.		$^{\circ}$ C.		
1-25-48	8,674	99	130-1	negative	0
1-30-48	3,980	100		negative	0
2- 5-48	2,000			negative	0
2- 7-48	1,890				
2- 8-48	1,610	98		negative	0
2-14-48	1,465			negative	0
2-16-48	3,062	98			
2-19-48	4,260				
2-20-48	3,780				
2-24-48	5,150	100	130-1	negative	0
2-25-48	6,468			negative	
2-27-48	4,959				
3- 3-48	6,177	99	130	negative	0
3- 6-48	4,940				0
4-20-48	206 γ % (vol. unknown)				

Bile containing duodenal contents were obtained on 2-8-48. These exhibited intense red fluorescence and were found to have a coproporphyrin concentration of 128 γ /100 cc. of which 82% was the type III isomer.

The erythrocyte protoporphyrin on 2-10-48 was 36 γ /100 cc. of cells. No porphyrin could be demonstrated in the plasma.

DISCUSSION

The two cases which have been described are of a type hitherto unrecognized. They are important

TABLE V
Feces porphyrin findings

Date	Copro-porphyrin	% type III isomer	M.P. of cryst. methyl ester	Uroporphyrin
	$\gamma/100$ Gm.		$^{\circ}$ C.	
2-10-48	32,800	90	—	0
2-15-48	39,600	90	130-1 $^{\circ}$	0
2-16-48	40,200	—	—	0

from a number of standpoints, one of which was referred to in paper I of this series (7), which dealt with the normal coproporphyrin of the urine. It is evident that if an individual such as one of the two present cases contributed to a pool being collected from presumably normal individuals, as in Grotepass' study (13), the result would be affected to a very marked degree and would be interpreted erroneously. The complete lack of symptoms in these two individuals is of much interest since it casts considerable doubt on the belief that the symptoms of lead poisoning and porphyria are "porphyrinopathic," as Carrié and others have believed. In porphyria, of course, compounds other than coproporphyrin III may well be implicated, but in lead poisoning one finds only coproporphyrin III. It appears, however, that there is one striking difference between the present cases and cases of lead poisoning, in so far as porphyrin excretion is concerned. But slight or no increases of coproporphyrin III are encountered in the feces in lead poisoning³ even though relatively large amounts are present in the urine. In the present cases the amounts in the urine were considerably larger than those usually encountered in lead or other heavy metal poisoning, while the fecal coproporphyrin III was roughly ten times that of the urine. Thus it is evident that the total production of coproporphyrin in these two individuals is on a much larger scale than that of the secondary coproporphyrinurias of ordinary type. This would support the belief that the disturbance in the present cases represents an "inborn error of metabolism," albeit one that differs fundamentally from that of ordinary porphyria, in which uroporphyrin or uro-type porphyrins are also found.

In Table V it is noted that 90% of the fecal coproporphyrin was type III. This would indicate that 10%, or from 3280-4020 γ /100 Gm., was

³ At least in chronic cases. We have not had opportunity to study the fecal coproporphyrin in cases of acute plumbism.

type I. This value would exceed the normal by approximately ten-fold. In the crystallization of the methyl ester, no evidence was observed for such an amount of copro-I as this figure would indicate. The isomer percentage values cannot be regarded as absolute, simply providing an approximation of the relative amounts of the two isomers. This is particularly true when either isomer constitutes more than 85% of the mixture (6b). If the value had been 98–99% type III, as in the case of the urine, a normal amount of type I isomer would have been indicated. As it is, a moderate increase cannot be excluded.

SUMMARY AND CONCLUSIONS

1. Two cases are described in which a great increase of coproporphyrin III excretion was unaccompanied by symptoms or signs of porphyria, metal or chemical poisoning or liver disease. The first case was that of a chemist who had had exposure to heavy metals and chemicals, of doubtful significance. The second was a negro laborer who suffered from mild diphtheria at the outset of the study, but whose heightened porphyrin excretion persisted for months after the diphtheria had disappeared.

2. Careful studies of several members of the family of the first case yielded no evidence of any disturbance of porphyrin metabolism. In the second case no other members of the family were available for study.

3. The duodenal contents in each instance contained large amounts of coproporphyrin III, presumably contributed by the bile. The feces contained corresponding increases, ranging from 32 to 69 mgm./100 Gm., from 10 to 20 times the amount in the urine. It is emphasized that this is quite unlike the ratio in chronic lead poisoning, in which considerably more coproporphyrin III is found in urine than in feces.

4. In neither case did the urine contain porphobilinogen or uro-type porphyrins.

5. The blood serum or plasma in the two instances was free of demonstrable porphyrin. The erythrocyte protoporphyrin was not significantly elevated in either case.

6. The general significance and probable constitutional nature of these cases is discussed.

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INTRA-ARTICULAR TEMPERATURE AS A MEASURE OF JOINT REACTION

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Clinicians studying Rheumatic Diseases have long used the surface temperature over the joints as indicative of the degree of synovial reaction. Many of the terms used in rheumatology imply temperature changes in the joint tissues, viz. "the acute hot joint of gout," "the cold swelling of chronic rheumatoid arthritis," the so-called "friction effect" or "heating-up" of osteoarthritic joints following exercise, or "articular jelling" (implying excessive joint cooling and stiffening on prolonged rest). Many measurements have been made of the skin temperature over diseased joints, but no study on intra-articular temperatures in human joints has been reported. Several studies (1-4) have been made of intra-articular temperatures in dogs and one study (5) was performed on the stifle joint of a horse to determine the coefficient of friction within the joint, with and without synovial fluid.

METHOD

In an effort to find a method for more accurate evaluation of activity of joint disease, and for determination of the effects of rest, exercise, physical therapy, drugs, and Roentgen rays on joints, the following technique for determining intra-articular temperature has been developed.

Temperature studies were performed on the joints of normal volunteers and arthritic patients. All the studies were carried out in a room having a mean temperature of 24° C. The subjects were reclining quietly for at least one half hour prior to the studies. Because of the ease of approach, the knee joint was the usual site of study. The skin was sterilized with an antiseptic (merthiolate) solution. The skin and joint capsule at a point one half inch medial to the medial border of the patella and high enough to avoid the fat pad was infiltrated with several cubic centimeters of 1% procaine solution. A 19 gauge needle was inserted through the synovial membrane into the joint space under the patella. Even in most normal joints, a few drops of fluid could be aspirated into a dry syringe. When it was certain that the point of the needle was under the patella and in the joint space, the

syringe was removed and a fine copper constantan thermocouple or a thermistor was threaded through the needle to a depth of several inches in order to project freely into the joint space. The needle then was either left in place or, more frequently, removed leaving the temperature-sensitive element in place. The emf developed by the thermocouples was measured by a Leeds and Northrup type K potentiometer, and the change in resistance of the thermistor was measured with a Wheatstone bridge. Thermocouples or thermistors were also fixed on the skin over-lying the patella, and in most cases a thermocouple was also placed on the skin of the opposite knee. Control temperature readings were taken after the joint had been at rest, with the element in place, for at least one half hour. If a therapeutic procedure was employed to modify joint temperature which would not permit leaving the metal thermocouple in place, it was removed. Immediately after the treatment, another aspiration and insertion was made at the same site in order to determine the magnitude of the elevation and to obtain a measure of the rate of cooling.

RESULTS

The amount of synovial fluid present appeared to make little difference in the joint temperature except to alter the rate of change. The position of the temperature-sensitive element in the joint was relatively unimportant. The thermocouple or thermistor often was inserted a distance of three or more inches into the joint space. Temperature measurements made during withdrawal of the element showed no appreciable variation until the measuring element was out of the joint. Thirty-one determinations of the internal temperature of the knee joint and the over-lying skin surface have been obtained on 25 individuals. Two studies were made of the elbow joint in one patient. In no instance was the rectal temperature elevated at the time of study. Four subjects were essentially normal except for possible age changes (all were between 35 and 42 years). Twelve patients had rheumatoid arthritis. Seven had degenerative joint disease; one had Reiter's syndrome and one had gouty arthritis. The degree of local clinical

¹ Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I

Temperatures obtained in the joint cavity and on the surface over the joint

Case	Diagnosis	Age	Sex	Relative activity	Temperature, °F.	
					Joint	Surface
1	Normal	35	M		91.1	86.2
2	Normal	38	M		88.6	87.5
3	Normal	44	M		90.7	89.5
4	Normal	32	M		90.8	85.2
5	Degenerative Arth.	54	F	Moderate	93.4	89.0
6	Degenerative Arth.	60	F	Moderate	94.2	90.9
7	Degenerative Arth.	48	F	Questionable	93.4	89.9
8	Degenerative Arth.	51	F	Questionable	93.7	90.8
9	Degenerative Arth.	67	F	Questionable	93.0	86.0
10	Degenerative Arth.	60	F	Questionable	95.6	90.8
11	Degenerative Arth.	52	F	Questionable	94.6	92.3
12	Reiter's Syndrome	21	M	Severe	93.3	89.7
				Moderate	90.6	90.8
				Slight	89.4	87.4
13	Chronic Gout	36	M	Moderate	96.0	89.2
					96.1	90.0

activity in the joint tested was estimated by the presence and severity of the following: swelling, tenderness, effusion and limitation of motion by pain.

The values obtained are presented in Tables I and II. The correlation ($r = 0.65$) of joint temperature with surface temperature was generally good, but there were notable exceptions. In one patient the joint temperature was as much as 7.6°F . higher than the skin temperature, while in two others the surface temperature was higher than the intra-articular temperature. Relatively few normal joints have as yet been studied to determine the variations in resting temperatures. The difficulty of obtaining volunteers has somewhat hampered this evaluation. However, skin temperatures thus far obtained in "normal" subjects range from 85.2°F . to 89.5°F . and joint temperatures from 88.6°F . to 91.1°F .

In degenerative joint disease clinical activity was difficult to evaluate. Patients with this disease had, in most cases, normal skin temperatures but their joint temperatures were all moderately elevated. In the cases of chronic gout and Reiter's syndrome the skin temperatures were normal although joint temperatures were definitely elevated.¹ The clinical improvement in the patient

¹ A knee joint temperature of 98.6°F . was observed recently in a patient in an active stage of gout.

with Reiter's syndrome was reflected in the lowered joint temperatures. In rheumatoid arthritis the highest joint temperature, 97.1°F ., was recorded in an elbow joint. The highest surface temperature was 95°F . In case No. 25 where the rheumatoid process was completely quiescent, both the skin and joint temperatures were subnormal. In rheumatoid arthritis there was a positive correlation of both surface and joint temperature with relative clinical activity (Figure 1). The correlation appears to be somewhat better with joint rather than with surface temperature. In one individual the surface temperature was higher than the joint (Table II).

A variety of physical measures were employed to modify the joint temperature. Although results will be reported in detail in a subsequent paper (6), some observations of interest, primarily in reference to the joint reaction itself, are reported herein. Fever therapy, induced by a pyrogen injected intravenously, resulted in increased joint and sur-

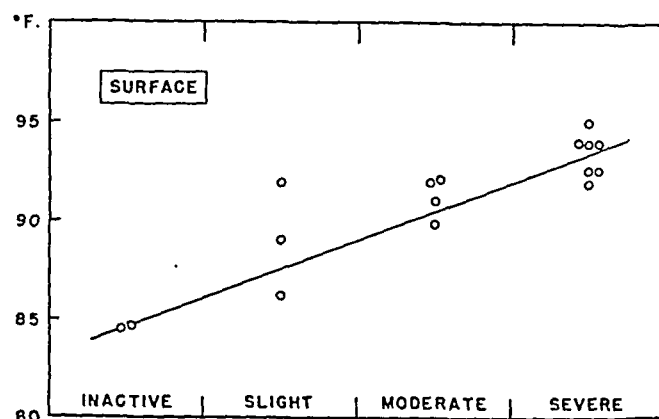
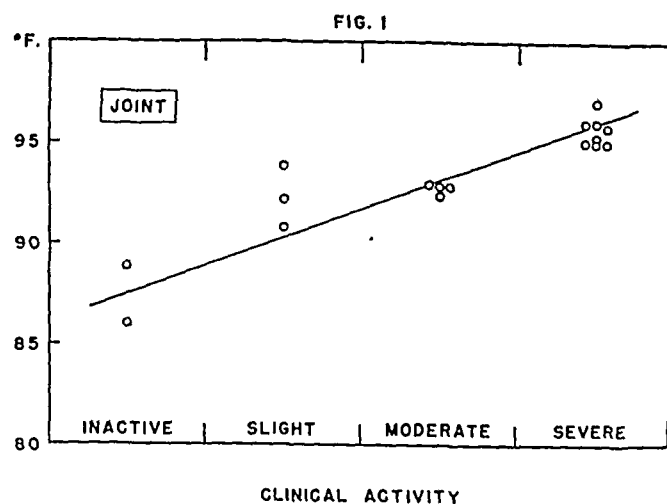


FIG. 1. TEMPERATURE OF JOINT AND SKIN OVER JOINT IN PATIENTS HAVING RHEUMATOID ARTHRITIS WITH VARYING DEGREES OF CLINICAL ACTIVITY

TABLE II

Temperatures obtained in the joint cavity and on the surface over the joint of patients with rheumatoid arthritis

Case	Age	Sex	Relative activity	Temperature, ° F.	
				Joint	Surface
14	56	F	Severe	95.0	93.7
				96.0	94.0
15	42	M	Severe	95.1	91.1
				95.3	92.6
16*	65	M	Severe	97.1	—
				95.0	95.0
17	18	F	Severe	95.8	92.6
18	40	F	Severe	96.0	93.9
			Moderate	92.8	92.1
19	61	M	Moderate	92.9	91.9
20†	30	M	Moderate	92.8	91.0
21	61	M	Moderate	92.4	89.8
22	68	F	Slight	93.8	86.2
23	50	M	Slight	90.8	91.9
24	71	M	Slight	92.2	89.0
25	57	M	Inactive	88.8	84.5
				86.0	84.7

* Elbow joint (all others were knee joints).

† Atypical.

face temperatures. When rectal temperature was elevated to 103° F., a joint temperature of 101° F. (an increase of 5.2° F.) and a skin temperature of 99.4° F. (an increase of 8.3° F.) was noted.

On another occasion, with a rectal temperature of 105.2° F. a joint temperature of 97.1° F. was obtained, but at this time the patient was being cooled by application of ice bags to the thighs. Even under these circumstances, the knee joint temperature was elevated 2° F. over the control observation.

Observations were made on the influence of hot and cold packs on both normal and inflamed joints. The application of *hot* packs invariably resulted in a depression of the intra-articular temperature of as much as 2.2° F. Continued reapplication of these hot packs produced steadily diminishing effects, although even after the tenth hot pack of a series, the joint temperature was still definitely lowered. The internal temperature quickly returned to control levels after discontinuation of the packs and five to ten minutes later the values were slightly higher, a positive reflex effect (Figure 2). Cold packs, on the other hand, raised joint temperatures appreciably. Hot or cold packs applied to one knee produced reflex effects in the opposite knee of similar character. These studies were performed in the winter months. When observations were resumed during the summer, it was surpris-

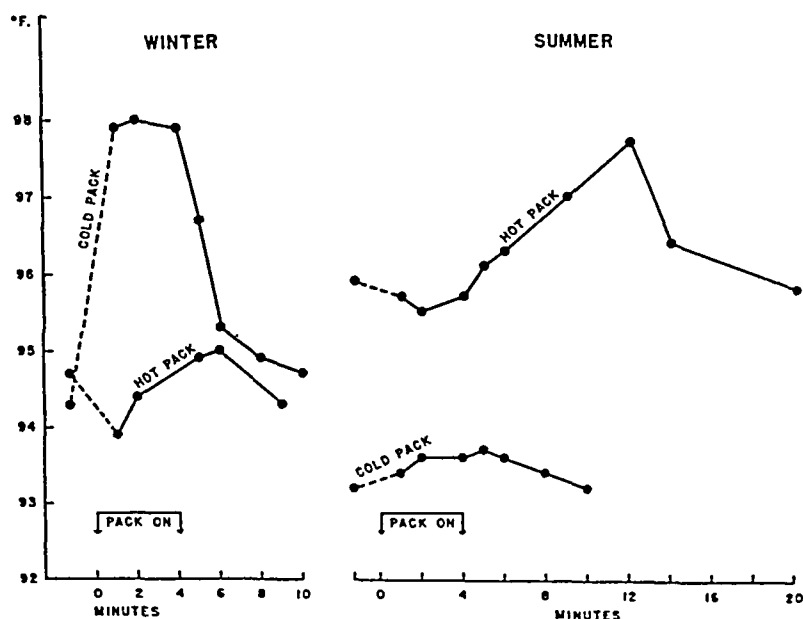


FIG. 2. REFLEX PHENOMENA ASSOCIATED WITH THE APPLICATION OF HOT AND COLD PACKS OVER THE JOINT

All points indicate joint temperatures. The dotted lines indicate the shift of the temperature from control values during the first minute of pack application.

TABLE III

Seasonal influence on the temperature reaction of the knee joint to hot or cold wet compresses. Maximum changes occurred within the first two of the five minutes during which the packs were applied

	Exp.	Type of pack	Maximum change, ° F.	
			Knee joint	Surface
Winter	1	Hot (108° F.)	-2.3	12.2
	2	Hot	-0.8	5.1
	3	Cold (65° F.)	2.5	- 9.1
	4	Cold	5.0	-10.1
	5	Cold	2.6	- 6.1
Summer	6	Hot	-0.4	6.4
	7	Hot	-0.5	5.8
	8	Hot	-0.5	5.1
	9	Hot	0.2	7.5
	10	Cold	0.5	- 3.6
	11	Cold	0.4	- 8.1

ing to note that although the pattern of the reflex was similar, the magnitude of the response was diminished (Figure 2 and Table III). This difference seems to be related to the relative degree of vascular tone exhibited by individuals at the various seasons of the year (7, 8).

Painful stimuli, apprehension, alarm or smoking lowered the skin and elevated the joint temperature in all cases so studied. Simple passive and non-weight-bearing active movement of the joint was performed in several instances. Temperature increases of as much as 2.5° F. were noted. The joint temperature following periods of active exercise showed varying degrees of elevation with rapid cooling on rest.

DISCUSSION

These preliminary observations of joint tissue temperatures in some of the forms of arthritis appear to indicate that an accurate evaluation of the degree of inflammation in a joint may be obtained by this method. A close correlation between the internal joint temperature and the clinical evaluation of activity of disease was not surprising and indicated that the temperature of the synovial fluid was directly dependent on the relative hyperemia of the synovium. However, the data would indicate that it is extremely difficult to evaluate the lower degree of clinical activity. The amount of heat "felt" externally over a joint was usually, but not necessarily, related to joint temperature. In degenerative joint disease, the elevation of joint

temperature noted seemed more closely correlated to the apprehension of the patient and the amount of adipose tissue about the knee than to the clinical activity as determined by external examination. The slow drop seen with continued rest in these patients would imply that this elevated temperature was real and was related to increased friction effect and retarded cooling.

Incidental observations were made on pain sensitivity of the synovium and cartilage, confirming the observations of Smyth and Freyberg (9, 10). The synovium was quite tender whenever touched at any point that had not been anesthetized with procaine. If the thermocouple was inserted far enough to touch the synovium on the opposite side of the patella, a sharp pain was produced. The articular cartilage, however, seemed entirely insensitive when touched, or even when accidentally penetrated by the aspirating needle or tip of the thermocouple.

In conjunction with the studies on the effectiveness of physical therapy procedures (6), one particularly interesting phenomenon was observed. Application of hot packs increased surface temperature markedly, but decreased the deep. A secondary rise of joint temperature occurred following removal of the packs. Cold packs to the knee surface, however, produced a definite cooling of the skin and a lasting increase in joint temperature. The intensity of hot and cold applications was not sufficient to induce pain in most instances but apparently this phenomenon is a sympathetic reflex effect associated with the principle of counter-irritation. The magnitude of this effect is apparently related to the state of vascular tone and dependent upon the season of year that observations were made (7, 8).

It appears significant that cold, pain, fear and smoking produced identical effects (*i.e.*, lowering of the skin and elevation of the joint temperatures). Perhaps this observation may lead to an explanation of how such factors aggravate the symptoms of an arthritic process. The effects of vaso-dilating and vaso-constricting drugs, deep X-ray therapy, exercise and other agents on the synovial circulation are being studied by this method. Finer measuring units have been developed which will make it possible to study temperatures in fingers, wrists, and other small joints.

No ill effects from these studies were noted in any of the patients. One study on a normal volunteer resulted in a slight synovitis of two days' duration, after weight-bearing exercise with the thermistor in place. Many of our patients expressed improvement after this "treatment," showing the psychic benefit derived from any robust procedure and the special interest exhibited by the physician.

SUMMARY

1. A method for the determination of the temperature of the articular cavity has been developed which appears to have a practical use both to measure the degree of inflammation in the joint and to determine the effect of external influences on joints.

2. Intra-articular temperatures correlated somewhat more closely than the surface temperatures over the joint with the clinical activity of rheumatoid arthritis.

3. The joint temperatures in degenerative joint disease were higher than anticipated from relative clinical activity. This might be related to greater joint friction effects and to retarded cooling.

4. The joint temperature fell when hot packs were applied over the joint, and rose with the application of cold packs. The magnitude of these

responses was affected by seasonal influences being greater in the winter than the summer.

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III. THE FATE OF CITRATE IN ERYTHROBLASTOTIC INFANTS TREATED WITH EXCHANGE TRANSFUSION^{1, 2}

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Since the adoption of exchange transfusion as a method in the treatment of newborn infants with severe erythroblastosis (1-3) the question of the possible toxic effects of sodium citrate added to the administered blood, to prevent coagulation, has arisen (4). In this procedure from 85 to 95% of the infant's Rh-positive erythrocytes are replaced by the donor's Rh-negative erythrocytes depending upon the volume of blood that is administered and simultaneously removed (5). In order to effectuate an exchange of 85%, a quantity of blood equivalent to twice the infant's blood volume must be used, and for an exchange of 98%, a quantity of blood equivalent to four times the blood volume must be used. Since the average newborn infant has a blood volume in the range of 250-300 ml. the procedure involves the use of approximately 500 ml. and approximately 1,000 ml., respectively. With the use of sodium citrate as an anticoagulant the infant receives as much as 60 to 120 ml. of a 3% solution of sodium citrate intravenously over a period of about 1½ hours.

In the experimental animal, the intravenous administration of large quantities of sodium citrate has been shown to produce convulsions (6). This has been ascribed to the formation of a poorly ionized calcium citrate complex which removes calcium ion from the blood stream (7-9). Despite this consideration, exchange transfusions have been successfully carried out in a large number of cases, although occasionally deaths have been reported in the very severely affected cases (10).

In order to study the adjustment that the newborn makes to the physiological strains to which it is subjected when an exchange transfusion is

carried out, citric acid, calcium, phosphorus, protein and total base levels were studied before, during and after such a transfusion on a newborn with no symptoms of erythroblastosis and with apparent normal hepatic and kidney function. The newborn studied was a mongolian idiot born in normal delivery. In addition citric acid determinations were carried out on serial bloods withdrawn from eight erythroblastotic infants during exchange transfusion.

500 ml. of blood were freshly drawn from a compatible donor. To this were added 60 ml. of 3% sodium citrate (dihydrate of tri-sodium citrate). This made a total volume of 560 ml. Over a period of approximately 60 minutes 500 ml. of this citrated blood were infused into the saphenous vein at the ankle. After 60 ml. had been infused the radial artery was cut and the blood was collected in 60 ml. portions, during the progress of the transfusion. The first 60 ml. were added without removal of blood in order to dilate the blood vessels and make the radial artery more apparent. The cells were separated by centrifuging and the plasma was analyzed for the various blood constituents.

Citrate determinations were carried out by a method previously described (11, 12). Calcium was done by direct precipitation from plasma (13) without ashing. Where citrate was at a high level the results were later shown to be significantly low because of the interference of citrate with calcium precipitation. The determinations could not be repeated with ashing subsequently because calcium salts were found precipitated in the plasma on standing. The calcium results were discarded and are not reported except where the citrate levels were low enough so as not to interfere, or where analysis was done by ashing on plasma from freshly drawn blood. Phosphorus was determined colorimetrically (14), total protein by a modified biuret method (15) and total base by electrodialysis (16).

The results obtained on the blood of the donor with added citrate, and on that of the newborn with no symptoms of erythroblastosis are shown before and after the transfusion in Table I. The results are averages of duplicates. Standards and recovery of added amounts of the constituents

¹ The third in a series on the mechanism controlling citric acid levels in the blood. The first two papers in this series appeared in the Journal of Clinical Investigation, Vol. 27, pages 446 and 450, 1948.

² This paper was presented at the Washington meeting of the American Chemical Society, August 30, 1948.

TABLE I

Comparison of citrated adult blood plasma with that of the blood plasma of the infant before and after the exchange transfusion

Sample	Phosphorus	Total base	Total protein	Calcium	Citric acid
	mg. %	meq./l.	%	mg. %	mg. %
Citrated Adult Plasma	3.7	178	6.7	9.8*	340 †
Infant's Pre-Transfusion	7.2	150	5.14	10.8	2.9
Infant's Post-Transfusion (one hour)	6.4	148	5.48	10.0	6.69
Infant's Post-Transfusion (19 hrs.)	—	—	4.6	10.3	1.47

* Determination on ashed plasma.

† Assuming a 40% hematocrit and no citric acid in the red cells the total citric acid added in 500 ml. of whole citrated blood would be: $5 \times 340 \times 0.6 = 1,020$ mg.

were also run. The results are in error by less than 5%. Calculations in the table assume no citrate in the red cells. Previous work indicates that in normal blood no significant amount of citric acid is found in the red cell (11).

Analysis for citric acid, phosphorus, protein and total base of the 60 ml. portions of the blood removed from the infant during the transfusion is shown in Table II. These determinations were done on plasma.

A chart showing the changing citric acid levels with time, for the infant with no symptoms of erythroblastosis, is plotted in Figure 1.

TABLE II

Analysis for citric acid, phosphorus, total base and protein on the plasma of the blood removed in 60 ml. portions during a 500 ml. exchange transfusion

Seven portions removed for a total of 420 ml.

Plasma sample (in order of removal)	Phosphorus	Total base	Total protein	Citric acid	Total citric acid removed in each 60 ml. sample of whole blood*
	mg. %	meq./l.	%	mg. %	mg.
1	7.5	—	—	12.3	4.4
2	7.6	—	5.21	25.4	9.2
3	6.8	—	—	19.0	6.4
4	6.9	—	5.24	22.4	8.2
5	6.4	—	5.34	32.2	11.6
6	6.4	155	5.41	34.4	12.4
7	6.3	158	5.81	42.8	15.9

Total Citric Acid Removed = 68.1 mg.

* Calculated by assuming a 40% hematocrit and no citric acid in the red cells. $0.6 \times \text{mg. \% in plasma} \times 0.6 = \text{total citric acid}$.

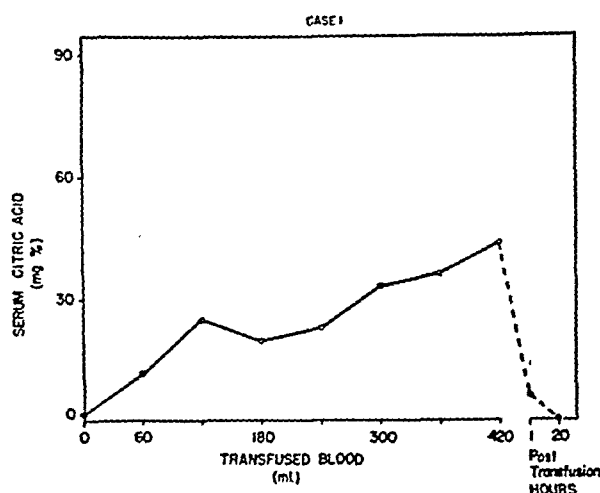


FIG. 1. CITRIC ACID LEVELS DURING AN EXCHANGE TRANSFUSION WITH CITRATED BLOOD ON A MONGOLOID INFANT WITH NO SYMPTOMS OF ERYTHROBLASTOSIS OR OF LIVER OR KIDNEY DAMAGE

In the eight cases of newborns with symptoms of erythroblastosis the determinations had to be limited to blood obtained during the transfusion. With the exception of Cases 2 and 6 the first sample obtained followed the administration of about 50–60 ml. of donor's blood and thus already contained added citrate. During the course of the transfusion, whenever symptoms of tetany or convulsions were impending or apparent, 5 ml. of a 10% solution of calcium gluconate were injected intravenously. In some instances, calcium gluconate was administered at various points in the procedure whether symptoms were or were not present.

The results obtained in six of these cases are shown in Figure 2. The scales of the graphs for Cases 3 and 4 are different with respect to citrate administered from that for Cases 5–8. In the first two cases 500 ml. of blood were infused, in the last four 1000 ml. of blood were used. Points at which calcium gluconate was administered are indicated with an arrow. These infants survived and have been followed for a period of at least six months and are all well. The details of their clinical course are incorporated in another report (17).

One infant in this series died within 24 hours after the transfusion. The changing citric acid levels for this infant are shown in Figure 3. On postmortem examination the liver of this child showed severe necrosis.

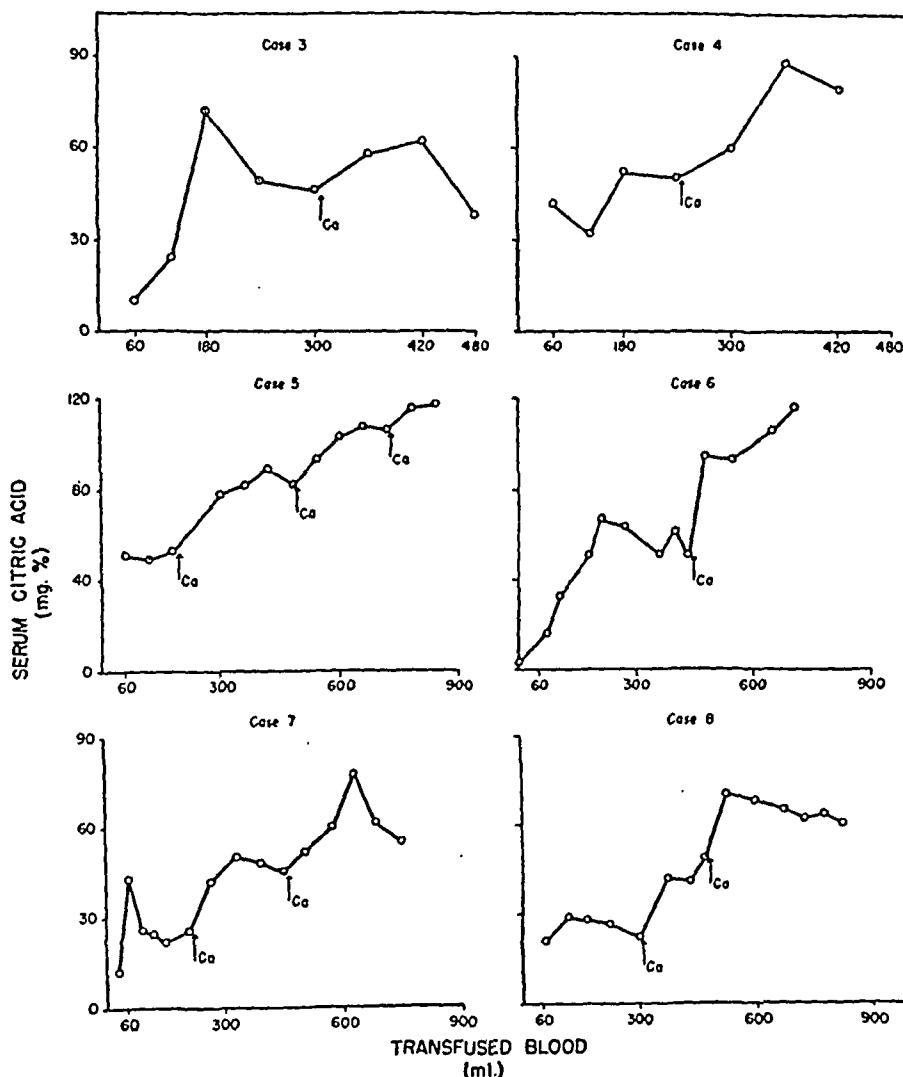


FIG. 2. CITRIC ACID LEVELS ON SIX ERYTHROBLASTOTIC INFANTS TREATED WITH EXCHANGE TRANSFUSIONS WITH CITRATED BLOOD. EFFECT OF ADMINISTRATION OF CALCIUM ON THE CITRATE LEVEL

Arrows indicate time of injection of calcium gluconate.

In view of the fact that we were unable to obtain the true total calcium values on the plasma of the infant with no symptoms of erythroblastosis fetalis, we studied the changing calcium values in a child with erythroblastosis fetalis during treatment with exchange transfusions. The bloods were dried at 100° C. and ashed in a muffle furnace at 475° C. before analysis so as to remove citrate interference. The results are shown in Figure 4 and Table III.

DISCUSSION

Examination of Tables I and II indicates a remarkable ability of the infant with no apparent liver or kidney damage to remove citric acid from the blood stream rapidly. After the addition of 60 ml. of citrated blood the citric acid level in the

plasma of the infant should rise, according to calculations, to approximately 60 mg.%.³ Actually, after the adding of the first 60 ml. of citrated blood, the level rises to 10 mg.%. In this infant the citric acid level never rose above 40 mg.% while

³ This figure is arrived at by assuming a 275 ml. blood volume and a hematocrit which is the same in the administered and endogenous blood. With a 40% hematocrit the infant's plasma volume would be 165 ml. For a 3 mg.% citric acid level in the plasma the total citric acid in this plasma would be 5 mg. Sixty ml. of citrated blood would contain 36 ml. of plasma with a citric acid concentration of 340 mg.% as determined. The citric acid content of this plasma would be 122.5 mg.%. The total citric acid in the combined volume of blood plasma (165 + 36 = 201 ml.) would then be 122.5 + 5 = 127.5 mg. Expressed in mg.% this would be 127.5/2.01 = 63.5 mg.%.

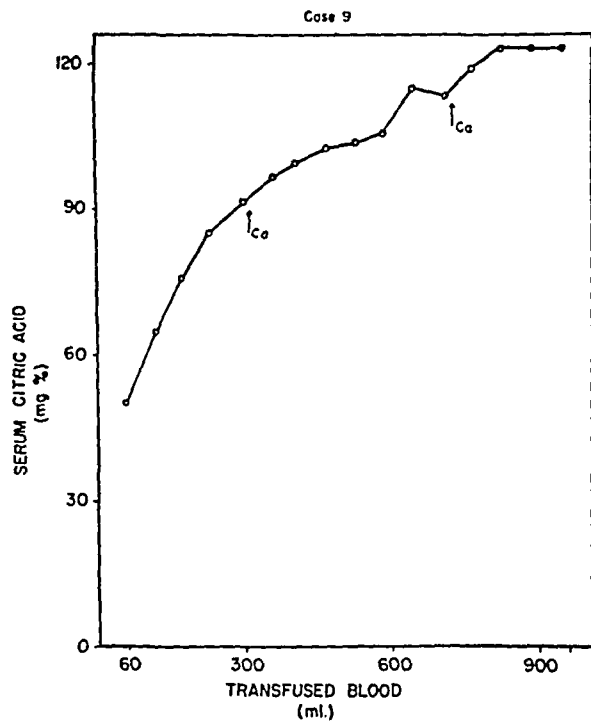


FIG. 3. CITRIC ACID LEVELS DURING AN EXCHANGE TRANSFUSION ON AN ERYTHROBLASTOTIC INFANT WHO DID NOT SURVIVE FOR MORE THAN 48 HOURS AFTER THE TRANSFUSION

Arrows indicate time of calcium gluconate injection.

TABLE III

Calcium determination on ashed blood plasma removed from an infant with symptoms of erythroblastosis during an exchange transfusion (see Figure 4)

Amt. of blood transfused	Pre-transfusion	60	120	180	240*	300	360	420*	480
Calcium† mg. %	10.0	12.8	14.5	15.3	24.0	21.0	15.4	19.0	17.0

* After 5 ml. of 10% calcium gluconate were added.

† Determined on ashed plasma.

the blood infused contained a concentration of 340 mg.%. According to the equation derived by Wiener (5), at the end of a 500-ml. transfusion 85% of the infant's blood has been replaced by the blood of the donor. At the end of a 1000 ml. transfusion 98% of the infant's blood has been replaced. As early as one hour after the termination of the procedure the level had dropped to almost the fasting level. The fasting level for newborn is of the order of 2-3 mg.% as reported previously (18, 19). A sample taken 19 hours later showed a level within the normal range.

In the last column of Table II are listed the actual quantities of citric acid that were removed from the infants at intervals during the procedure.

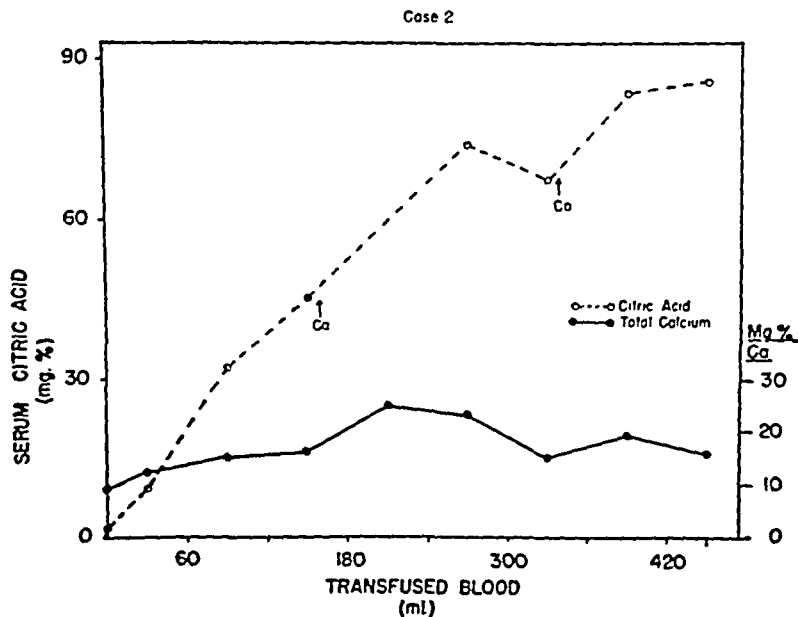


FIG. 4. CITRIC ACID AND TOTAL CALCIUM LEVELS DURING AN EXCHANGE TRANSFUSION ON AN ERYTHROBLASTOTIC INFANT

Arrows indicate time of injection of calcium gluconate.

These total 68 mg. as contrasted with 1,020 mg. infused. The infant retained 952 mg. This would indicate a retention of approximately 94% of the citrate injected. Only 71 mg. remained in the blood stream at the end of the procedure when the plasma level was 42.6 mg.%. This is calculated on the assumption of a volume of 275 ml. and a hematocrit of 40%.

Examination of Figures 2-4 indicates that the ability to lower citric acid levels in the plasma is somewhat impaired in the erythroblastotic infants transfused. Cases 4, 5, 7 and 9 show an immediate elevation of citric acid levels after the administration of the first 60 ml. of citrated blood, to a level of more than 30 mg.%. Case 9, who died, showed the highest level at this point (52 mg.%). It is of interest to compare these curves with that of the infant who showed no symptoms of erythroblastosis. Comparison can only be made on the part of the curve obtained before the administration of calcium gluconate, since injection of this material causes changes which will be discussed below.

Each point on the curves represents the addition of 60 ml. of citrated blood. All cases, with the exception of the infant who died (Figure 3) showed spontaneous lowering of citric acid levels in the face of continued administration of citrate. Thus all the cases which survived, with the exception of Case 5, showed lowering of citric acid levels after the initial rise, indicating a more rapid removal at these points than the rate at which the citrate was added. It is of interest to note that Case 5 clinically was the most severely affected of those who survived. Yet it is apparent that this infant's response to the administration of citrate clearly showed a less impaired mechanism than that of the infant who died (Case 9). For example, after 180 ml. of citrated blood had been infused (approximately two-thirds the infant's original blood volume) the citrate level was at 50 mg.%. At this same point in the transfusion Case 9 was at 78 mg.%. Case 9 showed little resistance to the rising citric acid levels. Similar comparison of Case 9 with all the other cases clearly showed the lesser ability of this infant to lower plasma citric acid levels.

The fate of administered citrate is suggested by several experimental studies on animals. Rabbits injected with sodium citrate subcutaneously ex-

hibit an increase in the size of the livers (20). This increase has been demonstrated to be due in part to an increase in liver glycogen following the administration of citric acid (21). It would appear, therefore, that in these cases of exchange transfusion the citric acid may be rapidly converted to glycogen and perhaps lipids.

That the major portion of this conversion takes place in the liver has been shown by the work of Sjöström (22). When livers of rabbits, dogs and cats were perfused with fluids containing sodium citrate in concentrations one hundred times as large as those found in normal sera, after a single passage the perfusate had a citrate concentration near to normal levels for that animal. When rabbit livers were then poisoned by allyl formate, the level of serum citrate rose and the perfused liver showed a markedly diminished ability to metabolize citrate. In confirmation of this, it has been found (23) that the concentration of citrate in the portal vein was regularly higher than that in the arterial blood.

The liver is, however, not the only organ involved in the removal of citrate from the blood stream. The kidney removes and concentrates citric acid. The level of citrate in the urine is approximately 15-50 times that of the serum (11). The concentration of citrate in the renal vein is 20% lower than that in the renal artery (23). Citrate is also metabolized in the musculature. Stoppani (24) has demonstrated rapid metabolism of injected citric acid in eviscerated dogs.

In conjunction with the clinical symptoms and in the light of autopsy findings on children who have died of erythroblastosis fetalis the observations on the lessened tolerance of erythroblastotic infants to administration of citrate may be traced to partial liver damage and perhaps concomitant kidney damage. The liver damage in the infant who died was so extensive, as observed on postmortem examination, that very little resistance could be offered to the rising citric acid values. We postulate, from their ability to remove administered citric acid from the blood stream, that the other infants who showed greater resistance to a rise in citric acid plasma levels, conversely, had lesser liver damage and therefore a greater chance of survival.

At all points where calcium gluconate was injected (Cases 2-9) a rise in citrate level followed.

In this connection it is of interest that injection of calcium salts, or of parathormone to raise the calcium level causes a rise in endogenous citrate levels (25).

That administration of calcium gluconate seems to interfere with the removal of citrate from the blood plasma must not be construed, for obvious reasons, as counterindicating the use of calcium gluconate to prevent tetany during the procedure.

In the case where calcium values were studied during the transfusion (Figure 4) a rise in endogenous calcium was noted before administration of calcium gluconate. This probably comes from the body stores of calcium (skeleton). The response is remarkably rapid. Studies on sheep (26) have shown that injection of citrate causes a rise in serum calcium which drops to normal after the citrate has been removed from the blood stream. Steggerda (27) has shown that oral administration of citric acid will cause a retention of calcium in humans followed later by increase in excretion in the stools. Similarly, it is probably from the bone stores that the phosphorus values are maintained in the face of the continuous administration of citrated blood containing half the inorganic phosphorus found in the infant's serum. The rise of calcium would therefore represent an endogenous mechanism for combatting the lowering of calcium ion concentration when citrate is infused. For the usual exchange transfusion, especially those involving the transfusion of 1,000 ml. of blood this needs to be supplemented by injection of calcium gluconate.

These findings may explain the clinical observations of numerous investigators on the use of citrate as the anticoagulant. Clinical experience with citrate in transfusions seems to indicate that it is a non-toxic anticoagulant (28). Further, large doses of citrate fed to animals over an extended period of time produced no toxic effects (29). The liver, the kidneys, the peripheral musculature and the bone stores all serve to lower citric acid levels or to neutralize their effect on calcium and magnesium ion concentration.

A marked disparity exists between the concentration of various blood constituents in the adult as compared with the newborn. Some of these differences are further enhanced by addition of sodium citrate to adult blood. Other than the change in citrate concentration the addition of 60 ml. of

3% sodium citrate to 500 ml. of blood will raise the total base of the plasma from approximately 150 meq./l to about 175 meq./l (determined as 178 meq./l).⁴ Table I compares the values for phosphorus, protein, total base, calcium and citric acid of the citrated adult blood with the blood plasma of the newborn with no symptoms of erythroblastosis.

Despite the fact that blood with a total base of 178 meq./l was administered rapidly and "flushed through" the circulatory system the total base of the infant's blood remained within the normal range (145-155 meq./l). The protein rise was small and only apparent toward the end of the procedure. One hour after the procedure was ended the protein values were falling, they were below the original fasting level of the newborn 19 hours later. The fall in protein and the appearance of a mild jaundice at the end of 19 hours may indicate some liver damage. Within 48 hours after the procedure the infant showed no untoward effects. Phosphorus levels during and at the end of the transfusion remained close to that of the infant's pre-transfusion blood rather than assuming the phosphorus level of the donor's blood.

From the observations above it is apparent that citrate as an anticoagulant has advantages. When infused, as in an exchange transfusion, into the newborn it is readily metabolized and removed from the blood stream restoring the natural clotting capacity of the blood within one hour after the transfusion is complete. However, a reasonable amount of liver and kidney function must be present to make possible the removal of citrate, the readjustment of total base, protein and phosphorus levels to their natural level for the newborn.

Using the icterus index and low protein level as criteria, observed in the control 19 hours after the transfusion, it must be said that some temporary

⁴ 500 ml. of adult blood would contain 300 ml. of plasma assuming a hematocrit of 40%. With a total base of 150 meq./l, 300 ml. of plasma would contain 45 meq. The molecular weight of the dihydrate of sodium citrate is 295. For our purposes it may be assumed to be 300 so that a 3% solution would contain 100 millimoles/l or 300 meq./l. Sixty ml. of this solution would contain 18 meq. The total plasma volume of the 560 ml. of citrated blood would then be $300 \div 60 = 360$ ml. In this volume would be contained $45 \div 18 = 63$ meq. Expressed in meq./l the citrated plasma would contain 175 meq./l.

liver damage is produced in this process. However, in the infant with no symptoms of erythroblastosis recovery was observed 48 hours after the transfusion with return to normal icterus and protein level.

SUMMARY

1. Exchange transfusions were carried out on one mongoloid newborn with no symptoms of liver damage or erythroblastosis and on eight newborns suffering from erythroblastosis fetalis.

2. Serial studies done on the blood removed from the normal infant showed an effective homeostatic mechanism to be operative in the case of citrate, total base, phosphorus and total protein.

3. Administration of calcium gluconate interferes with the rate of citrate removal from the blood stream.

4. The liver, kidneys, musculature and bone stores appear to remove or neutralize the effect of high citrate levels in the blood stream.

5. The erythroblastotic infants showed impaired ability to remove citrate but showed themselves still capable of removing massive quantities of citrate from the blood stream.

6. With the exception of one case all infants seemed to metabolize citric acid more rapidly or at least as rapidly as it was infused in the early stages of the transfusion. In this case where the disease process was most severe citric acid levels rose steadily throughout the transfusion. The infant died within 24 hours.

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ELECTROLYTE CHANGES IN HUMAN STRIATED MUSCLE IN ACIDOSIS AND ALKALOSIS

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INTRODUCTION

In recent years many studies have been reported on the monovalent cation composition of the intracellular space during experimentally produced potassium depletion. The most consistent changes have been found in striated muscle and, in general, have shown that decreased concentrations of intracellular potassium are associated with an increase in intracellular sodium. Such changes have been reported in the rat (1), the cat (2), and the dog (2) following low potassium diets. The administration of desoxycorticosterone acetate in large doses has produced similar changes in the same animals (2-4). Recently Darrow has reported comparable findings in association with alkalosis, as determined by muscle analyses in rats (5) and indirectly by balance studies in man (6).

Inasmuch as direct studies of human tissues during states of potassium depletion have not been reported, the present studies were undertaken in an attempt to define the composition of the intracellular electrolytes of striated muscle obtained by biopsy from patients with metabolic alkalosis and acidosis.

METHODS

Biopsies. Muscle biopsies were taken from the hamstring or gastrocnemius muscles, the average weight of fresh tissue being 2.4 gms. Anesthesia was obtained by infiltration of the skin and subcutaneous tissues with 2 per cent procaine in saline. Careful precautions were taken to avoid infiltration of the muscle tissue with the anesthetic agent and it is believed that there was no contamination of the specimens with the possible exception of two cases (Table I, patients G. and To.). In these patients inadequate anesthesia necessitated infiltration of the muscle approximately 10 cm. proximal to the site from which the actual biopsy was taken. A careful analysis of the results obtained from these two specimens reveals no abnormality specifically attributable to the possible inclusion of the anesthetic solution in the specimen, and it is

therefore believed that no contamination occurred. Excess blood was removed by blotting the specimen with dry gauze. The tissue was immediately placed in an airtight glass container and analytical procedures were begun within one hour. Venous blood specimens without stasis were taken under oil at the time of biopsy.

"Normal" control muscle specimens and accompanying blood samples were obtained from patients undergoing operations under general anesthesia (Table I), the biopsies being taken from the rectus abdominis at the start of the operation. The patients had been on a normal diet, had not received previous parenteral alimentation, and were considered to be in a normal state of fluid and electrolyte balance. The alleged normalcy of the control muscles may be subject to question because of possible changes resulting from the anesthetic agents or the underlying disease process. Minor changes may also have resulted from sustaining infusions of glucose and saline started at the time of operation. However, the "normal" values obtained are in essential agreement with previously reported analyses of human skeletal muscle (7), and because of the marked differences between the "normal" and abnormal groups, it is felt that these tissues represent an adequate control for the present study.

Analytical Methods. Determinations were made of the concentration of water, fat, chloride, sodium, potassium, and nitrogen in the tissues. All analyses were done in duplicate. The analytical procedure was that of Lowry and Hastings (8) as modified by Hull (9), with additional modifications for determination of sodium and potassium by an internal standard flame photometer (10). The fresh tissue was freed of all visible fat and connective tissue and then finely minced with scissors and placed in a 10 cc. volumetric tube. The water content was measured by drying in an electric oven at 105-110° C. to constant weight. Neutral fat was determined by the extraction with ethyl ether of the residue remaining after the evaporation of water, according to the procedure of Hastings and Eichelberger (11). The extracted fat and solvents were returned to the tube and the solvents evaporated. The tissue was digested in a bath of boiling water with 3 ml. concentrated nitric acid in the presence of silver nitrate 50 per cent in excess of anticipated chloride. Chloride content was determined by Volhard titration of an aliquot of the digest made up to volume with water. Sodium and potassium were determined on 1 or 2 cc. aliquots diluted to the proper volume with the addition of lithium sulfate as an internal standard. Flame photometry yielded an accuracy of 1 per cent, and large

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excesses of nitric acid and inorganic phosphate added to standard solutions introduced no measurable error. Nitrogen estimations were done on samples of tissue separate from those taken for electrolyte analysis, and nitrogen was determined by micro-Kjeldahl technique. All analyses were corrected for fat-free weight, including nitrogen estimations for which it was assumed that the fat content was the same as in the aliquot used for electrolyte determinations. Duplicate analyses of separate aliquots of minced muscle from 42 specimens analyzed by this method have shown the following average percentile deviation from the mean values: water $\pm 0.2\%$; chloride $\pm 1.9\%$; sodium $\pm 1.5\%$; potassium $\pm 1.2\%$; and nitrogen $\pm 0.9\%$.

Serum electrolytes were determined by methods previously reported (3), except that sodium and potassium were determined by flame photometry. Serum pH was determined by glass electrode.

Calculations. Serum concentrations were corrected for serum water which was either determined directly by drying to constant weight at 110°C ., or estimated from serum protein concentration (8). Extracellular concentrations of sodium, potassium and chloride were calculated using an average Donnan factor of 0.95. Extracellular water (ECW) was calculated as the "chloride space." Intracellular electrolytes were obtained as the difference between tissue and extracellular values and have been expressed in concentrations per liter of intracellular water (ICW). Because tissue hemoglobin, collagen and elastin were not measured, all tissue nitrogen was considered as intracellular, thus introducing a small systematic error of less than 1 per cent (8). For comparative purposes nitrogen has been expressed as grams per liter ICW.

RESULTS

Nine patients were studied—three controls, three with gastric alkalosis, and three with chronic renal acidosis without glomerular insufficiency (12). The results are given in Table I. The last two analyses of patient R. have been excluded from the present discussion because blood specimens were not taken simultaneously with the muscle. Calculated intracellular sodium averaged 8 mEq/L ICW in the control group. There is no apparent explanation for the variation of the values within the control group. Both the patients with acidosis and alkalosis showed an increase of intracellular sodium, average values being 32 mEq/L ICW for the acidosis group and 20 mEq/L ICW in alkalosis. Serum potassium varied from 3.9 to 4.8 mEq/L for the controls, from 2.5 to 3.7 mEq/L for the acidotic group, and from 1.8 to 2.7 mEq/L for the alkalotic group. The increase in intracellular sodium was proportional to the decrease

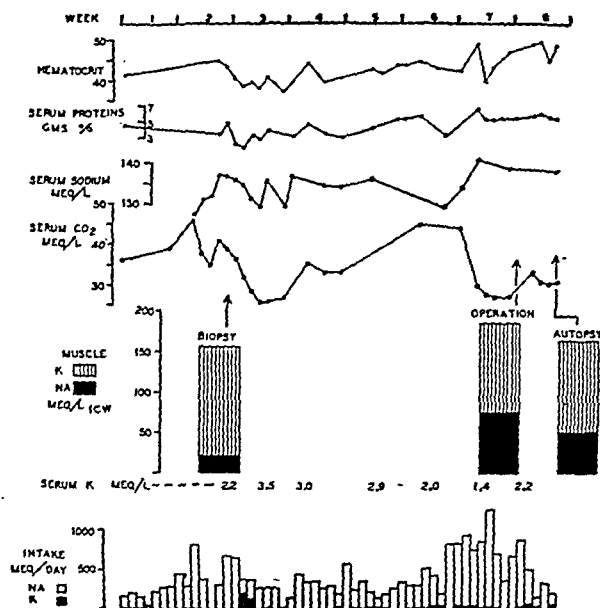


FIG. 1. CLINICAL COURSE, SERUM ELECTROLYTES, AND INTRACELLULAR ELECTROLYTES IN PATIENT R. SHOWING DEVELOPMENT OF INTRACELLULAR POTASSIUM DEPLETION WITH SODIUM REPLACEMENT AS PROLONGED ALKALOSIS WAS TREATED WITH PARENTERAL SODIUM CHLORIDE SOLUTIONS

in intracellular potassium as is indicated by the constancy of the sum of the concentrations of these two cations, average values being 165 mEq/L ICW for the controls, 163 mEq/L ICW for the acidotic subjects and 163 mEq/L ICW for the patients with alkalosis. The widest variations of these values was found in the group with alkalosis. The ratio of nitrogen to cell water indicates a relative cellular dehydration in acidosis with a slight increase in hydration in alkalosis. However, the changes in cellular hydration are small and are not definitely conclusive.

The most striking abnormalities were found in the serial analyses of patient R. (Table I and Figure 1) who had persistent vomiting due to a carcinoma of the duodenum. During 63 days he received 239 L of parenteral fluids, therapy being primarily directed towards the maintenance of a normal hematocrit and normal concentrations of serum sodium, chloride and protein. Random analyses revealed a continuous loss of potassium in vomitus and urine. No parenteral potassium was administered. Although the calculation of the intracellular space in the last two muscle analyses can only be accepted as an approximation because

TABLE I-A

PATIENT	AGE	SEX	DIAGNOSIS	SPECIMEN	ANESTHESIA	SERUM CONCENTRATIONS									
						H ₂ O	Protein	HCO ₃	Cl	Na	K	Ca	P	Urea N	pH
						%	%	mEq/L	mEq/L	mEq/L	mEq/L	mEq %	mEq %	mEq %	
T	48	M	Normal	Rectus Abd	Pentothal, H ₂ O, Curare	91.2*	7.5	27.7	96.8	133	4.0	-	-	9	-
Th	62	F	Normal	Rectus Abd	Pentothal, H ₂ O, Curare	91.5	6.8	22.4	99.2	137	3.9	-	-	11	-
M	32	M	Normal	Lat. Dorsi	Pentothal, H ₂ O, Curare	90.6	7.4	24.2	94.6	134	4.8	-	-	-	-
D	46	M	Renal Acidosis	Gastroc.	Procaine, local	91.0*	7.5	13.5	116.8	137	3.7	9.3	2.2	16	-
P	28	F	Renal Acidosis	Gastroc.	Procaine, local	90.2	7.9	12.8	114.2	135	2.9	8.3	2.3	18	7.25 V
G	28	F	Renal Acidosis	Gastroc.	Procaine, local	90.2	7.8	11.6	113.0	138	2.5	9.5	2.2	20	7.28 A
K	57	F	Gastric Alkalosis	Hamstring	Procaine, nerve block	92.7	6.5	49.0	62.8	128	1.8	9.9	3.5	43	7.62 V
To	63	M	Gastric Alkalosis	Gastroc.	Procaine, local	92.4	6.2	43.2	82.8	137	2.7	-	-	17	7.52 V
R-1	65	M	Gastric Alkalosis	Gastroc.	Procaine, local*	92.7*	5.8	39.2	87.6	137	2.2	9.3	2.7	26	7.53 V
R-2				Rectus Abd.	Pentothal, H ₂ O, Curare	92.9*	5.6	32.9	93.0	139	1.4	-	-	-	-
R-3				Pecus	Autopsy	93.1*	5.4	31.0	84.3	139	2.3	-	-	94	-

* - Calculated from Serum Protein

V - Venous Blood without Stasis

A - Arterial Blood

TABLE I-B

PATIENT	MUSCLE COMPOSITION						CALCULATED VALUES						REMARKS
	Fat	H ₂ O	N	Cl	Na	A	ECW	ICW	Na	K	Na plus K	N	
	%	gm	gm	mEq	mEq	mEq	ml	ml	mEq	mEq	mEq	gm	
	fresh tissue	per Kg fat-free tissue					per Kg fat-free tissue		per liter intracellular water				
T	1.0	793	32.1	28.1	39.1	87.0	251	542	8	159	167	59.3	Carcinoma of Stomach.
Th	7.1	802	29.3	29.7	44.2	81.9	259	543	14	149	163	54.0	Carcinoma of Rectum.
M	2.8	764	33.7	27.4	35.9	88.0	248	536	2	162	164	62.8	Hypertension without cardiac or renal complications.
D	4.3	778	34.2	38.5	48.7	74.5	284	494	17	149	166	69.2	Osteomalacia. Muscular atrophy and recurrent fasciculations. Deep reflexes hyperactive. Staggering gait and easy fatigability. No paralysis.
P	4.2	761	34.5	25.1	49.1	69.1	189	572	39	120	159	60.3	Osteomalacia. Recurrent diffuse muscle pains, slight fatigability. Deep reflexes hyperactive. No paralysis.
G	3.5	766	37.4	30.9	55.3	66.1	235	531	40	124	164	70.3	Osteomalacia. Generalized paralysis of voluntary muscles, respiration and swallowing unimpaired. Blood pressure and pulse normal. ECG - isoelectric T1, low T2 and T4P, depression R lead II. Normal deep tendon reflexes. Complete disappearance of paralysis after parenteral and oral KCl. Subsequent ECG normal.
K	7.4	783	33.7	14.2	41.3	85.7	198	585	26	146	172	57.6	Vomiting for 3 weeks. Moderate dehydration. Signs of latent tetany. ECG - premature contractions, QT interval 0.45 seconds. ECG normal after recovery from alkalosis.
To	10.1	794	30.8	24.4	43.3	80.5	258	536	13	149	162	57.4	Persistent vomiting for 2 weeks. No latent tetany.
R-1	2.9	787	32.0	19.3	39.7	80.9	194	593	21	136	157	55.9	Jan 31. Vomiting for 3 weeks. No signs of latent tetany even after hyperventilation. No paralysis. ECG normal.
R-2	1.3	783	31.9	19.3	70.1	66.1	184	599	74	120	184	53.2	March 8. Operation - resection of Carcinoma of Duodenum. Received parenteral alimentation for high intestinal obstruction. Enesis averaged 1.5 L per day. Oral intake restricted, parenteral fluids averaged 4 L per day, mostly saline. Received 13 transfusions, no other source of potassium intake. Serum values obtained 18 hrs. before operation.
R-3	1.6	828	26.7	36.0	75.1	51.4	377	451	48	112	160	59.5	March 13. Autopsy. Death preceded by cardiac failure, edema, uremia, and jaundice. No signs of latent tetany and no signs of myasthenia or paralysis during entire hospital course. Serum value obtained 6 hrs. before death.

the blood and muscle specimens were not simultaneous, the muscle specimens obtained at operation and autopsy showed, respectively, that 40 per cent and 31 per cent of the intracellular cation was sodium. The serum values for the autopsy

specimen were obtained six hours before death, the autopsy being performed 11 hours post mortem. The extent to which post mortem changes may have modified the calculated intracellular values cannot be accurately evaluated at present.

DISCUSSION

The calculation of the extracellular and intracellular spaces and the electrolytes contained herein has been based on the concept either that all chloride is extracellular (11, 13, 14), or that a small but invariable amount of chloride is intracellular (15). Because of inadequate data on human muscle, the possibility of a small constant quantity of chloride within cells has been neglected. However, the obligatory extracellular position of chloride has been challenged by recent experiments in which large doses of potassium chloride were administered (16, 17). An analysis of the results reported here indicates that the chloride content of whole muscle varies directly with the serum chloride concentration. Small deviations from an exactly linear relationship may so easily be explained by slight variations in the extracellular fluid volume that we are led to believe that there is no significant alteration in muscle cell permeability to the chloride ion during disturbances of the type reported here. We therefore feel that the use of the chloride space as a measure of the extracellular space is valid for these studies.

Although the number of specimens is too small to warrant definitive statistical analysis, it is apparent in the conditions studied that there has been a loss of intracellular potassium from striated muscle with an increase in intracellular sodium. The human, therefore, responds in a manner qualitatively similar to other species studied.

In recent studies Darrow *et al.* (18) have demonstrated a correlation between alkalosis and a rise in intracellular sodium in the presence of normal renal function. In acidosis produced by the administration of ammonium chloride to rats, they found a decrease in the amount of sodium within striated muscle cells. Although our studies of acidosis have been limited to a single disease, namely, chronic renal acidosis without apparent glomerular insufficiency, the fact that these patients demonstrate a significant depletion of intracellular potassium and a rise in intracellular sodium in the presence of systemic acidosis indicates that these changes are not necessarily associated with alkalosis of the body fluids. Albright *et al.* (12) showed that this type of renal abnormality was associated with a negative potassium balance, which has been confirmed by

unreported balance studies of two of our patients. These findings, therefore, suggest that the described abnormalities in the intracellular electrolyte pattern may not be specifically related to changes in acid-base equilibria, but rather are a reflection of an overall negative potassium balance, regardless of the immediate cause.

The relationship of disturbances in potassium metabolism to paralysis has been recently reviewed (19). Of our six cases with documented potassium depletion, only one patient developed paralysis (subject G., Table I). On the day of biopsy the patient was unable to walk, feed herself, or turn over in bed. The paralysis had developed gradually during the preceding days and had not been precipitated by a large carbohydrate intake. Serum potassium was 2.5 mEq/L, a value of 2.6 having been obtained on the second and sixth day before the episode of paralysis. Following the administration of parenteral and oral potassium chloride the patient rapidly improved with disappearance of all symptoms of paralysis. During the following two weeks of observation, the serum potassium ranged from 2.8 to 3.2 mEq/L. Although a spontaneous remission cannot be absolutely ruled out, the fact that the paralysis disappeared following the administration of potassium chloride is evidence that the disturbed neuromuscular function was actually caused by a potassium deficit. It is of particular interest, therefore, that the concentrations of potassium in the serum and muscle of this patient were not significantly different from those of the others studied. The findings in patient P. (Table I) were almost identical, yet she had no paralysis. On the other hand, patient R. (Table I) showed the greatest potassium depletion, but throughout his hospital course had no manifest disturbances of neuromuscular function. Our limited observations thus fail to reveal any correlation between potassium concentration within skeletal muscle and the appearance of paralysis.

CONCLUSIONS

1. The association of alkalosis with decreased concentrations of intracellular potassium and increased concentrations of intracellular sodium has been confirmed by analysis of biopsies of human striated muscle.

2. Similar changes have been observed in patients with systemic acidosis due to chronic renal disease.

3. One patient with severe paralysis attributed to potassium deficit showed intracellular changes which were quantitatively not different from the non-paralyzed patients.

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STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, SERUM IRON, SERUM IRON-BINDING CAPACITY AND PLASMA COPPER DURING NORMAL PREGNANCY¹

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Changes in the free erythrocyte protoporphyrin, plasma iron, serum iron-binding capacity and plasma copper in normal individuals and in various diseases associated with anemia have been investigated previously in this laboratory (1). The present observations were carried out to determine the "chemical pattern" of the blood in the pregnant state.

METHODS

The hematological methods used have been described previously (2). Free erythrocyte protoporphyrin was measured according to the method of Grinstein and Watson (3). The method of Barkan and Walker (4) was used for the measurement of serum iron. The iron-binding capacity of the serum was determined by a modification (5) of the method of Schade and Caroline (6). Plasma copper was determined according to the procedure of Cartwright, Jones and Wintrobe (7).

Eighty-six pregnant women were studied. The determinations were made at monthly intervals during pregnancy and the postpartum period. Thirty-one women were studied only once, 32 were followed for two months, 15 for three months, five for four months, and three for five months. In the entire study 139 determinations of packed red cells were made, 89 erythrocyte protoporphyrin determinations, 137 serum iron determinations, 129 plasma copper determinations, and 74 determinations of the serum iron-binding capacity. Sixteen women received ferrous sulfate by mouth during a part of the study. The values in this group showed no significant deviation from those in patients not receiving specific therapy. Nevertheless, the values obtained during the period of iron administration have not been included in the data presented in the figures. Placental blood was collected from the umbilical vein immediately after delivery of the placenta.

In this laboratory, the following are the values found in normal females: serum iron $104.3 \pm 36.4 \mu\text{g}$ per cent, iron-binding capacity of the serum $371 \pm 35.9 \mu\text{g}$ per cent, plasma copper $122.7 \pm 10.6 \mu\text{g}$ per cent (1). Observed values for free erythrocyte protoporphyrin have shown a skew distribution and the geometric mean \pm two standard deviations has been found to be 34 (14 to 83)

$\mu\text{g}/100$ ml. of red blood cells. The normal subjects were not usually examined completely but they were apparently healthy and the hematologic determinations including the red cell indices were within normal limits in all instances.

RESULTS

Volume of packed red cells. The changes in the volume of packed red cells during pregnancy can be seen in Figure 1. During the first trimester, there was little deviation from the normal mean.

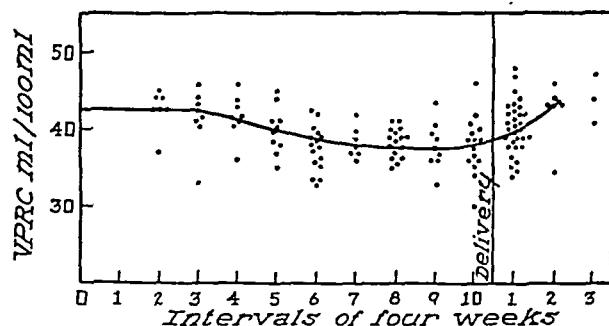


FIG. 1. VOLUME OF PACKED RED CELLS (VPRC) DURING PREGNANCY AND THE POSTPARTUM PERIOD

This was followed by a gradual decrease in the percentage of packed red cells. The lowest values were obtained in the last trimester, during the latter half of which the mean was found to be 37.9 ml./100 ml. as compared with 42.5 during the first trimester. During the postpartum period a return to values found in non-pregnant women took place within the first two months.

Erythrocyte protoporphyrin. The trend of the free erythrocyte protoporphyrin during pregnancy can be seen in Figure 2. There appeared to be a slight increase in the mean value during each successive month but only five determinations were above, and only slightly above, the upper limit of normal.

Analysis of the relationship of the free erythrocyte protoporphyrin and the mean corpuscular

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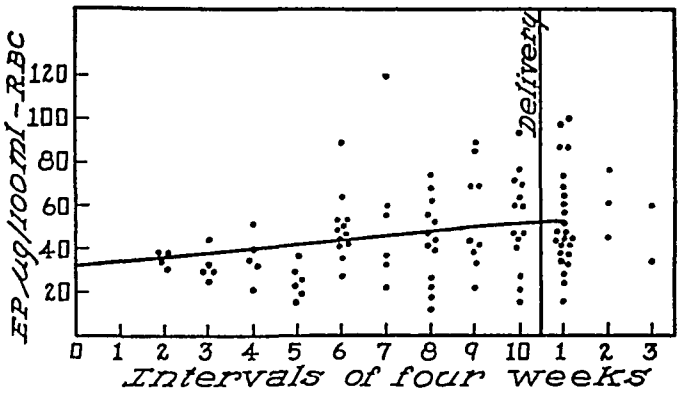


FIG. 2. FREE ERYTHROCYTE PROTOPORPHYRIN (EP) DURING PREGNANCY AND THE POSTPARTUM PERIOD

hemoglobin concentration during the last trimester of pregnancy, reveals a correlation coefficient of -0.54 . However, since the mean corpuscular hemoglobin concentration ranged only from 31 to 37 per cent with two-thirds of the values 33 or 34 per cent, this correlation cannot be regarded as significant.

Serum iron. In Figure 3, the values for serum iron are presented. In the first trimester, no change was observed. After this, there was a gradual decrease in the mean, reaching the lowest value in late pregnancy. Within the first three months of the postpartum period a return to normal occurred. There were wide individual variations at all stages.

Iron-binding capacity. The changes in the total iron-binding capacity of the serum are presented in Figure 4. The values during the first 20 weeks were found to be within normal limits. During the second half of pregnancy the iron-binding ca-

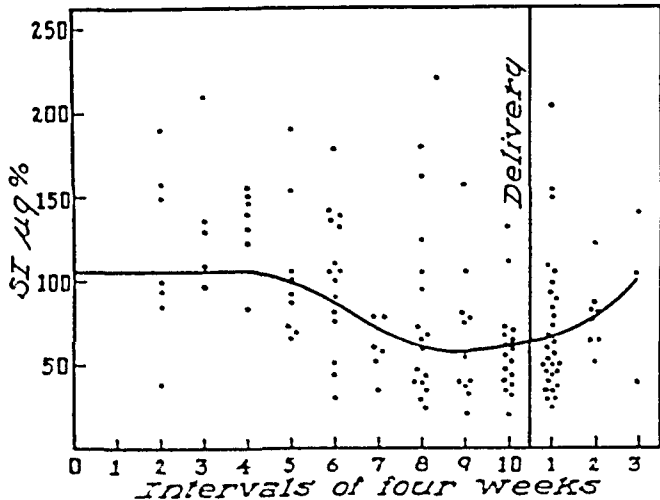


FIG. 3. SERUM IRON (SI) DURING PREGNANCY AND THE POSTPARTUM PERIOD

capacity increased; the highest values were found in the last trimester. Within eight weeks after delivery, normal values were again attained. It should be noted that the iron-binding capacity increased at the time the serum iron diminished and the iron-binding capacity curve is the reciprocal of the serum iron curve.

Plasma copper. The results of the determinations of plasma copper are shown in Figure 5. Of the chemical constituents studied this was the first to change during pregnancy. The copper content of the plasma became elevated early in pregnancy and increased steadily until the third trimester, during which period it remained at a maximum until delivery. During the first two months of the postpartum period the values returned to the normal level.

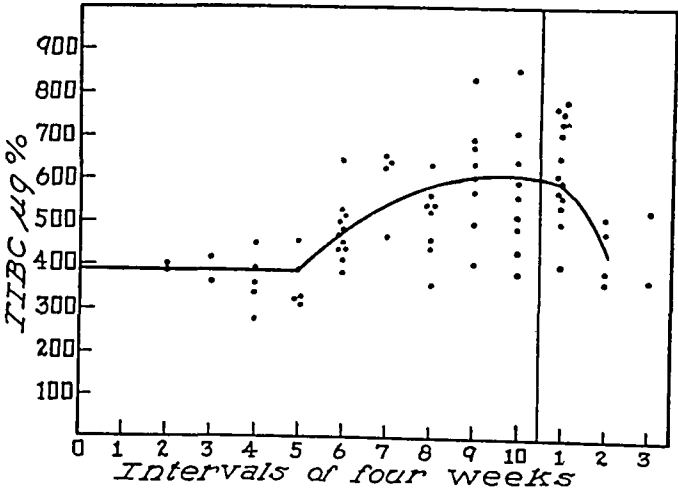


FIG. 4. TOTAL IRON-BINDING CAPACITY OF THE SERUM (TIBC) DURING PREGNANCY AND THE POSTPARTUM PERIOD

Placental blood. The placental and maternal serum iron and plasma copper are compared in Table I. The specimens were taken from the mothers at the time of delivery. As can be seen, the values for copper were considerably higher in the maternal blood than in the placental blood

TABLE I
Comparison of placental and maternal plasma copper and serum iron

Determination	No. of det.	Placental	Maternal
Plasma Copper $\mu\text{g per cent}$	14	75 ± 14	260 ± 42
Serum Iron $\mu\text{g per cent}$	7	164 ± 71	75 ± 32

TABLE II
Summary of the data

Determination	Normal females	40th week pregnancy
Volume Packed Red Cells ml./100 ml.	44.5 \pm 0.4	37.9 \pm 1.0
Erythrocyte Protoporphyrin μ g/100 ml. RBC	39 (16-70) *	53 (16-93) *
Serum Iron μ g per cent	104 \pm 5.5	59 \pm 6.4
Total Iron-Binding Capacity μ g per cent	371 \pm 9.3	583 \pm 50.3
Plasma Copper μ g per cent	123 \pm 1.5	261 \pm 10.5

Values represent mean \pm standard error of the mean.

* Since the frequency distribution curve is skewed an arbitrary range has been selected (1).

whereas the converse was true in regard to serum iron.

DISCUSSION

Certain alterations from the normal in copper, iron and iron-binding capacity of the serum have been observed during pregnancy, while no significant change has been found in free erythrocyte protoporphyrin. The results are summarized in Table II. The earliest change noted was an elevation in the plasma copper. This was followed in the middle of the second trimester by a decrease in the

serum iron and a corresponding increase in the serum iron-binding capacity. The greatest changes in all three of these values were observed in the last trimester. In Table III morphologic and chemical studies on 22 women during the last trimester of pregnancy are given in detail. These findings are in accord with those of others (8-13).

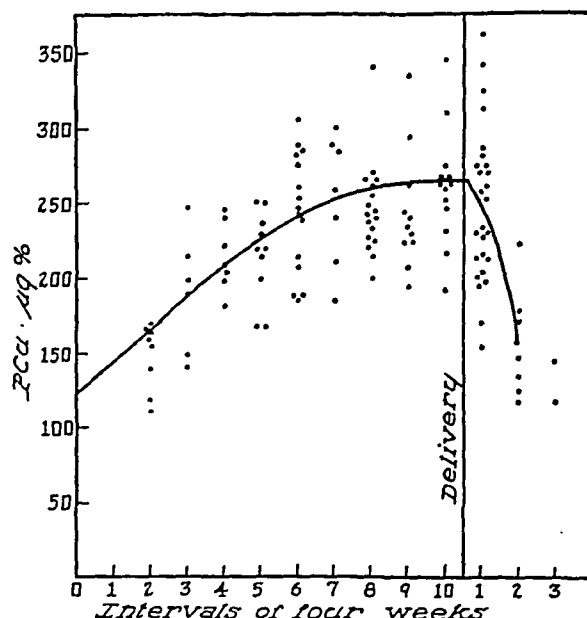


FIG. 5. PLASMA COPPER (PCU) DURING PREGNANCY AND THE POSTPARTUM PERIOD

TABLE III
Detailed morphologic and chemical studies on 22 pregnant women during the last trimester of pregnancy

Patient	Age	Week of pregnancy	RBC	Hgb	VPRC	MCV	MCH	MCHC	Plasma copper	Serum iron	Erythrocyte protoporphyrin
	Yrs.		millions/ c.mm.	gm. per cent	ml./100 ml.	C. μ	$\gamma\gamma$	per cent	μ g per cent	μ g per cent	μ g per cent
P. E.	20	30	4.34	10.8	35.0	81	25	31	226	42	68
L. J.	34	31	3.79	13.2	37.0	98	35	36	267	125	25
G. G.	17	32	4.24	13.6	41.0	97	32	33	248	44	54
L. M.	30	36	4.08	13.8	39.0	95	34	35	242	79	40
E. L.	34	31	4.15	11.9	35.6	86	29	33	224	30	44
G. E.	22	34	4.90	14.2	40.0	82	29	35	216	59	62
C. F.	26	40	4.74	13.1	41.0	87	28	32	274	40	36
M. S.	30	40	5.10	13.8	40.8	80	27	34	216	64	16
G. B.	26	40	4.33	10.8	35.4	82	25	31	346	55	60
A. C.	23	30	4.68	12.7	38.4	82	27	33	235	40	55
A. W.	30	31	4.18	12.4	37.6	90	30	33	237	158	12
L. L.	20	31	4.53	14.5	40.8	90	32	35	341	162	18
B. C.	21	40	4.35	12.9	40.6	93	30	32	311	69	28
H. H.	25	40	4.76	12.6	38.4	84	26	31	266	48	60
M. P.	21	39	4.37	12.6	38.4	88	29	33	253	40	47
N. L.	18	36	4.97	13.2	40.6	82	27	33	246	37	85
C. D.	29	31	4.29	13.5	36.0	84	31	37	222	94	39
E. R.	23	40	4.95	13.5	39.6	80	27	34	248	71	64
J. C.	31	30	4.79	12.8	39.0	80	27	33	220	54	56
R. W.	25	30	4.98	13.2	40.0	80	26	33	262	66	43
B. C.	24	40	4.32	13.6	40.4	93	31	34	214	154	29
J. N.	19	40	4.15	13.6	40.6	98	33	33	195	29	69

The changes noted during pregnancy differ in certain respects from those observed in various types of anemia (1). Thus, although as in the anemia associated with iron deficiency there was an increase in plasma copper, a decrease in serum iron and an increase in the iron-binding capacity of the serum, the serum iron values in pregnancy did not reach the extremely low levels seen in iron deficiency nor was there the marked increase in the free erythrocyte protoporphyrin seen in iron deficiency anemia. The chemical pattern does not correspond to that observed in the anemia of infection, where an increase in erythrocyte protoporphyrin and a decrease in iron-binding capacity of the serum occurs. Even in the presence of infection, the iron-binding capacity during pregnancy was found to remain elevated.

The increase in iron-binding capacity during the latter half of pregnancy occurs at a time when the demand for iron on the part of the fetus is greatest. The present observations reveal a close time correlation between the decrease in serum iron and the increase in iron-binding capacity. If the changes in these values were a function of variations in plasma volume, the deviations would of course be in the same direction. It may be that the elevation in the iron-binding capacity is part of the mechanism for increased iron absorption, mobilization and placental transfer and is analogous to the increase in iron-binding capacity of serum in iron-deficient states.

The elevation in plasma copper would seem to be independent of the changes in iron since there is no correlation in time or degree between these two values. One explanation that has been offered for the high values in the maternal blood is that the elevation of copper is related to the mechanism for mobilization of the element for placental transfer. This theory is based on the fact that copper storage in the tissues of the fetus, especially in the liver, is much greater than in the adult (14-16). It has been observed also that the copper content of placental arterial blood is greater than that of placental venous blood by 30 per cent (17). This suggests that there is a constant withdrawal of copper from the maternal blood via the placenta and storage in the fetus. It seems strange, however, that the high copper content of the fetal tissues should be associated with a low fetal plasma copper, while the high iron content of the fetal

tissues is accompanied by a high serum iron in the fetus and a low serum iron in the mother.

A relationship between the elevated basal metabolic rate in pregnancy and the increased plasma copper has been suggested (13). Hypercupremia has been noted in non-pregnant patients with increased metabolism (9, 18). An elevation in serum precipitable iodine has been observed during pregnancy (19) over the same period of time in which an elevation of plasma copper was found in this study. Both of these changes are evident before there is a change in the basal metabolic rate. It is possible that the elevation in plasma copper is related to changes in endocrine equilibrium during pregnancy. It is clear that additional knowledge concerning the metabolism and functions of copper is needed before conclusions can be drawn concerning its significance in various clinical conditions.

SUMMARY

The free erythrocyte protoporphyrin, serum iron, serum iron-binding capacity, and plasma copper have been measured in 86 normal pregnant women. A decrease in the volume of packed red cells was noted in the latter part of pregnancy. There was no significant change in the free erythrocyte protoporphyrin, although a slight elevation of the mean was found during the later phase of pregnancy. A diminution in serum iron was evident during the latter part of pregnancy and at the same time the iron-binding capacity of the serum increased. Plasma copper values increased during the first trimester and remained elevated during the course of pregnancy. Normal values were regained during the first two months postpartum. Study of placental blood revealed low values for plasma copper while the serum iron level was higher than the corresponding values in the maternal blood.

ACKNOWLEDGMENTS

We are indebted to Miss Betty Tatting, Mrs. Darlene Kehl, Miss Mary Iles and Miss Pauline Black for technical assistance.

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STUDIES ON THE ACTIVATION OF A SERUM "PROTHROMBIN-CONVERTING FACTOR"

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Recent reports of additional factors operating at the first stage of blood coagulation have stimulated investigators to explore in greater detail the mechanism by which prothrombin is converted to thrombin.

This report describes a new experimental approach to the problem of prothrombin conversion which, it is felt, may help to clear up some of the complexities of this reaction. In the present study, it will be shown that thromboplastin added to human serum is able to activate a substance which will be called a "prothrombin-converting factor." When thromboplastin is added to serum, the activated factor added to plasma then converts prothrombin to thrombin in the absence of ionized calcium. After initial activation of serum by thromboplastin, the "prothrombin-converting factor" undergoes gradual decay until the serum-thromboplastin mixture is no longer reactive. "Prothrombin-converting factor" can then be repeatedly reactivated from the same serum-thromboplastin mixture by addition of more thromboplastin. When serum obtained from patients treated with dicumarol is activated by thromboplastin, a more prolonged action of the released "prothrombin-converting factor" results.

The mechanism by which these observed reactions are brought about is discussed and a modification of current concepts relating to prothrombin conversion is proposed.

Investigators from different laboratories have utilized diverse methods to arrive at the conclusion that there exist in plasma previously unrecognized factors which initiate the conversion of prothrombin to thrombin. When Seegers *et al.* (1) studied purified prothrombin, they observed that a slowed rate of prothrombin conversion occurred after the reaction was initiated by adding thromboplastin and calcium. At first this retarded

conversion was thought to be related to denaturation of prothrombin attendant upon chemical procedures necessary for fractionation and purification. Later Ware *et al.* (2, 3) and Fantl and Nance (4) were able to produce acceleration of thrombin formation from this purified prothrombin fraction, when serum was added to the prothrombin, thromboplastin and calcium reaction mixture. The protein fraction responsible for accelerating prothrombin conversion was called accelerator globulin (ac-globulin). Ware *et al.* (3) believed that ac-globulin produced a reaction of co-autocatalysis, wherein thrombin accelerates its own formation through an intermediate action of ac-globulin. A minute amount of thrombin produced early in coagulation of blood, liberates accelerator globulin which then intensifies the rate of thrombin formation from prothrombin.

Milstone (5, 6) reported another plasma globulin fraction, prothrombokinase, which is capable of forming thrombin from prothrombin. Prothrombokinase is activated by calcium to form an active thrombokinase which then converts prothrombin to thrombin. Activation of the thrombokinase follows an autocatalytic reaction, requiring considerable time before maximum thrombokinase activity is reached.

Quick (7, 8) postulated the existence of prothrombin A and a labile factor to explain certain discrepancies occurring in prothrombin determinations made by his method. He found that certain plasmas with a low concentration of prothrombin could be restored to a higher concentration following the addition of de-prothrombinated plasma from a normal individual. These observations suggested that some plasmas might be deficient in prothrombin A or a labile factor.

On the basis of studies made on a patient with an unusual type of prothrombin deficiency that could likewise be restored by addition of de-pro-

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thrombinated plasma from a normal individual, Owren (9) postulated a different interpretation of results. He assumed that normal blood plasma contains an activator substance for prothrombin which he calls Factor VI. Factor VI exists as an inactive pro-enzyme Factor V which can be activated by thromboplastin and calcium in the presence of prothrombin to form Factor VI. Factor VI thereupon causes an enzymatic conversion of prothrombin to thrombin.

With these divergent interpretations and definitions in mind, it has seemed logical in the experiments to be reported, to speak of the factor in serum, which is activated following the addition of thromboplastin, as "prothrombin-converting factor." It may be that the "prothrombin-converting factor" is synonymous with Factor VI of Owren (9), thrombokinese of Milstone (6), prothrombin A and labile factor of Quick (7, 8) and accelerator globulin of Ware *et al.* (3).

EXPERIMENTAL METHODS

Collection of serum: Blood samples were collected from normal individuals and patients hospitalized for miscellaneous diseases. Dry syringes were used to obtain the blood and all collections were made as quickly as possible to avoid incipient coagulation. The plasma was prepared by adding 9.0 cc. of whole blood to 1.0 cc. of 0.1 M sodium oxalate solution. Serum was prepared by allowing whole blood to coagulate in glass tubes with internal dimensions of $\frac{5}{8} \times 6$ inches. Approximately 45 minutes later, the clot was rimmed with a wooden applicator and the serum, after centrifugation, was separated from the clot.

Preparation of thromboplastin: Dried thromboplastin was prepared according to Quick's method (10). Both human and rabbit brain preparations were utilized without any apparent difference in the results. Fresh thromboplastin suspension was made each day by mixing 0.3 gm. of dried brain powder with 5.0 cc. of 0.85 per cent sodium chloride solution. The thromboplastin suspension thus prepared was shaken periodically for 20 minutes at 50° C. after which, gross particles of brain were removed by centrifugation for three minutes at 1,000 R.P.M. Thromboplastin suspension prepared in this manner produced coagulation of recalcified, fresh, oxalated human plasma in 17–20 seconds at room temperature (22° C.).

Method of activating the serum "prothrombin-converting factor": Serum to be tested was allowed to remain at room temperature for two to three hours to allow destruction of thrombin activity to take place. Thrombin activity of serum was measured by adding 0.2 cc. of serum to 0.2 cc. of fresh plasma. Coagulation of the plasma usually did not occur until four or five minutes had elapsed. Serum capable of coagulating plasma in less

than four minutes was not used in the tests reported. Many of the sera were also tested for prothrombin activity by adding 0.1 cc. of serum to 0.1 cc. of fibrinogen and 0.1 cc. of thromboplastin suspension. The coagulation time of this mixture was usually greater than 180 seconds indicating that an insignificant amount of prothrombin remained.

Following these preliminary experiments to insure that the serum did not contain substances interfering with the test, activation of the "prothrombin-converting factor" was accomplished in the following manner:

Five-tenths cc. portions of serum were added to 0.1 cc. portions of thromboplastin suspension and thoroughly mixed. Simultaneously a stop-watch was started and at exactly one, three, seven, and 15 minute intervals, 0.1 cc. of the serum-thromboplastin (serum-TP) mixture was withdrawn into a 1.0 cc. pipette, calibrated to the tip, and blown into 0.1 cc. of plasma. The coagulation time of the serum-thromboplastin-plasma mixture was then determined to the nearest second. All of these operations were carried out at room temperature (22° C.). All coagulation tests were made in glass tubes with internal dimensions 12 × 25 mm. The coagulation time was determined as the point at which the plasma-serum-thromboplastin mixture first showed visible fibrin formation as the glass tube was tilted gently up and down from a diagonal position. A similar dilution of thromboplastin was made in 0.85 per cent sodium chloride solution to which sufficient calcium chloride had been added to produce a molarity of calcium ion corresponding to the ionized calcium of serum (0.0012 M). When this suspension was added to the plasma containing 0.01 M sodium oxalate, no coagulation occurred.

Since no calcium had been added to serum prior to the addition of thromboplastin, it is evident that the coagulating action of the serum-TP mixture could not be related to a direct effect of thromboplastin on the prothrombin of the plasma.

Effect of addition of thromboplastin to serum: The kinetics of the reaction taking place after addition of thromboplastin to serum are presented in Figure 1, which shows the results obtained from activation of sera from three normal individuals on three successive days. One minute after the introduction of 0.1 cc. of thromboplastin suspension to 0.5 cc. of serum, 0.1 cc. of the mixture added to 0.1 cc. of plasma produced coagulation in eight to 13 seconds. After three minutes incubation of the serum-TP mixture, plasma was coagulated in 12–19 seconds. Seven minutes' incubation of the serum resulted in plasma coagulation in 27–41 seconds following the addition of 0.1 cc. of this mixture to 0.1 cc. of plasma; while incubation for 15 minutes resulted in a plasma coagulation time of 50–80 seconds. If the serum-TP mixture was allowed to react several hours, no coagula-

COMPILATION OF RESULTS OF SERUM REACTION CURVES FROM THREE
NORMAL INDIVIDUALS ON THREE SUCCESSIVE DAYS

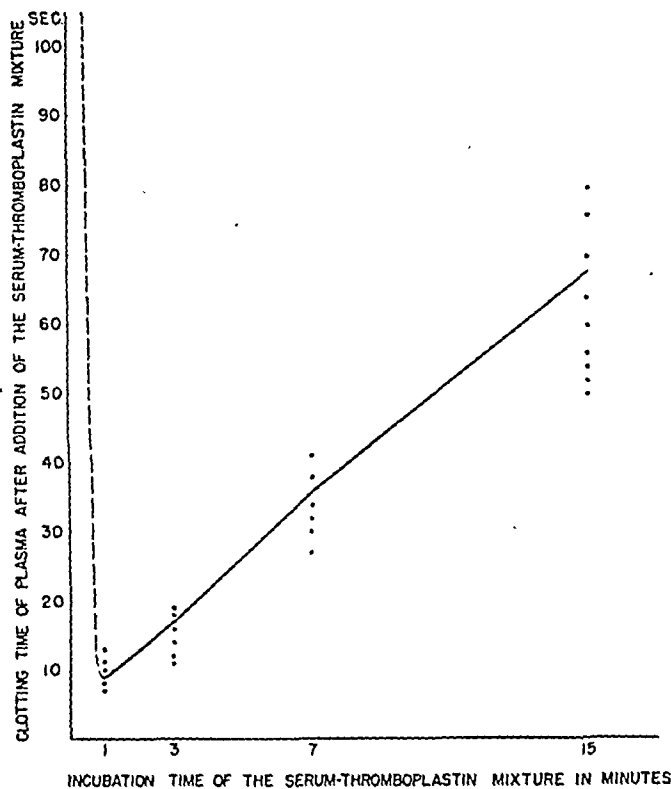


FIG. 1

The coagulation time of oxalated plasma, following the addition of serum-thromboplastin mixture, is indicated on the ordinate. The incubation time of the serum-thromboplastin mixture before it is added to oxalated plasma is indicated on the abscissa. Observe that maximum activation of serum occurs within one minute of the time of adding thromboplastin, following which, there is a slow decay of coagulating power of the serum-thromboplastin mixture.

tion of the plasma occurred when the serum-TP mixture was added to plasma. However, if 0.1 cc. of inactive serum-TP mixture was mixed with 0.1 cc. of plasma and 0.1 cc. of 0.02 M CaCl_2 then added, coagulation occurred as rapidly as when an identical amount of thromboplastin alone was added to plasma along with 0.02 M CaCl_2 . Furthermore, addition of the inactive serum-TP mixture to fresh serum caused activation of a "prothrombin-converting factor" to the same degree as thromboplastin correspondingly diluted in saline and mixed with serum. These experiments indicate that the thromboplastin is still active in the serum-TP mixture, but has apparently been altered in some manner so that activation of the "prothrombin-converting factor" no longer takes place.

If serum is activated by thromboplastin and then the mixture is added to either de-prothrombinated plasma or fibrinogen (Figures 2 and 3), little or no coagulation is produced, indicating that the mechanism of coagulation of the normal plasma cannot be related to a thrombin effect. A mixture of thromboplastin in 0.0012 M CaCl_2 in physiological saline which reproduces the molarity of ionized calcium in the serum, does not produce coagulation of plasma containing 0.01 M oxalate, so that one cannot relate the coagulating action of the serum-TP mixture to the thromboplastin and calcium content of the mixture.

EFFECT OF ADDING SERUM-THROMBOPLASTIN TO
FIBRINOGEN AND PROTHROMBIN

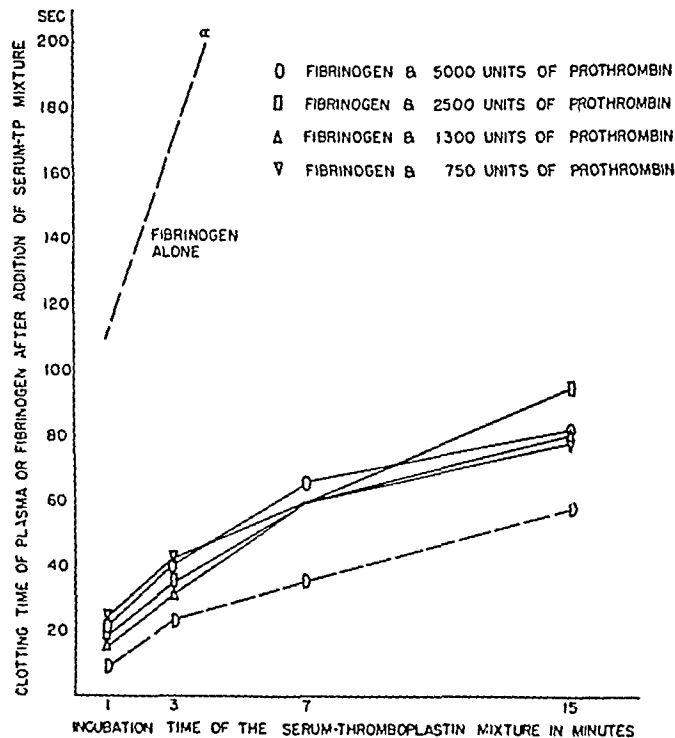


FIG. 2. EFFECT OF ADDING A SERUM-THROMBOPLASTIN MIXTURE TO FIBRINOGEN AND FIBRINOGEN-PURIFIED PROTHROMBIN SOLUTIONS

The ordinate indicates the coagulation time of the various fibrinogen preparations, following addition of serum-thromboplastin. The abscissa indicates the incubation time of the serum-thromboplastin mixture before it is added to the fibrinogen solutions. The uppermost broken line represents the results obtained when fibrinogen alone was used; while the solid lines show the results obtained when varying amounts of prothrombin were added to fibrinogen. The bottom broken line shows the effect of adding the same serum-thromboplastin mixture to fresh oxalated plasma containing a prothrombin concentration 100 per cent of normal.

The maximal coagulating action of the activated serum appears one minute after addition of thromboplastin. At this point plasma is coagulated much more rapidly than one would expect if one added only thromboplastin and calcium to plasma to determine the prothrombin time. From this evidence it can be assumed that addition of thromboplastin to serum initiates a reaction which causes liberation of a substance which in turn is capable of converting prothrombin to thrombin.

EFFECT OF VARYING CONCENTRATIONS OF PROTHROMBIN IN PLASMA

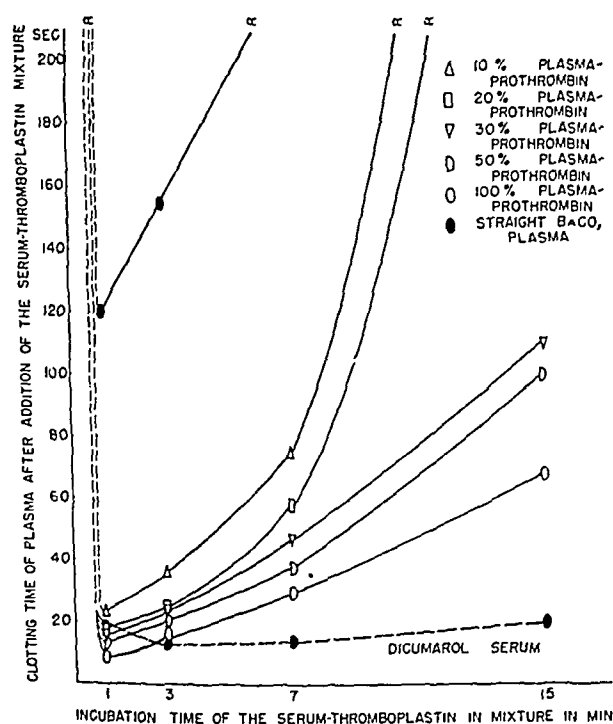


FIG. 3

Prothrombin was adsorbed from plasma with BaCO_3 . Aliquots of fresh plasma were then added to the BaCO_3 -treated plasma to produce the desired concentration of prothrombin. The coagulation time of plasma after addition of the serum-thromboplastin mixture (ordinate) was then plotted against the incubation time of the serum-thromboplastin mixture (abscissa). A decreasing concentration of prothrombin in the plasma is associated with a more abrupt decay of the activated serum's power to coagulate plasma. The broken-line curve was obtained by activating serum from a patient treated with dicumarol whose plasma prothrombin concentration was 20 per cent of normal. This serum, activated by thromboplastin, was then added to a BaCO_3 -treated plasma containing 10 per cent prothrombin. A striking difference in the activation curve is apparent.

REACTIVATION OF THE SERUM-THROMBOPLASTIN MIXTURE

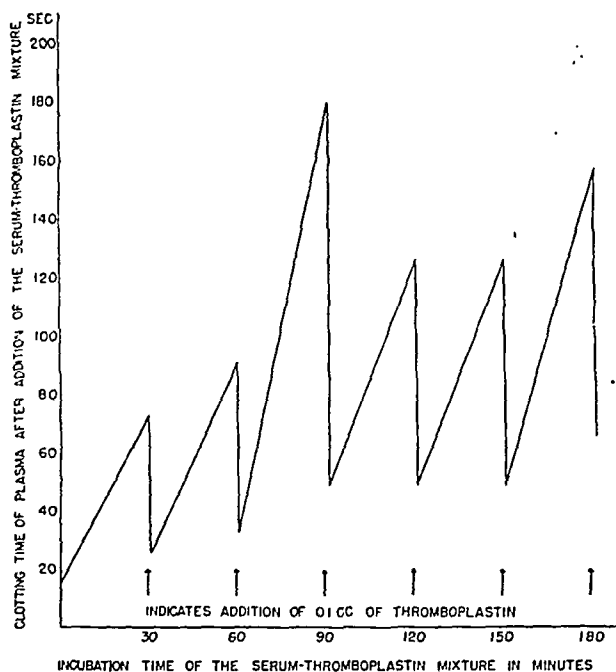


FIG. 4. REACTIVATION OF A SERUM-THROMBOPLASTIN MIXTURE BY INCREMENTS OF THROMBOPLASTIN

One cc. of serum was added to 0.2 cc. of thromboplastin. Thirty minutes later, 0.1 cc. of this mixture was added to 0.1 cc. of oxalated plasma and the coagulation time recorded. An additional 0.1 cc. of thromboplastin was then added to the initial serum-thromboplastin mixture and one minute later, 0.1 cc. of this was withdrawn and added to oxalated plasma. The curve of decay of the serum-thromboplastin coagulating substance is interrupted by addition of fresh thromboplastin. The serum-thromboplastin mixture can be repeatedly reactivated as shown in this graph.

The serum-thromboplastin mixture produces no coagulation of plasma, after a sufficient period of incubation of the serum and thromboplastin has taken place. Reactivation of the serum component can be produced, however, when fresh thromboplastin is added to the inactive serum-TP mixture. After serum and thromboplastin were allowed to react for 30 minutes, it was found that 0.1 cc. of this mixture produced coagulation of 0.1 cc. of plasma in 120 seconds. An additional 0.1 cc. of thromboplastin was then added to the above serum-TP mixture. One minute later 0.1 cc. of the new serum-TP mixture coagulated 0.1 cc. of plasma in 20 seconds, demonstrating that the clotting activity of the serum-TP mixture had been almost completely restored. As shown in Figure 4, alternating decay and reactivation were ob-

served many times. It appears that the activated serum factor capable of producing plasma coagulation is not destroyed but only reversibly inactivated. It can be reactivated repeatedly, merely by adding more thromboplastin to the serum.

Effect of adding activated serum to "artificial plasmas": The clotting activity of a "prothrombin-converting factor" of serum, activated by thromboplastin, was further studied by observing the effect of adding activated serum to fibrinogen solutions.

Human fibrinogen² was dissolved in 0.85 per cent sodium chloride solution (pH 7.5) to make a concentration of fibrinogen of 200–300 mg. per cent. It will be observed in Figure 2, that serum-TP mixture coagulated fibrinogen in 110 seconds in contrast to a coagulation time of 10 seconds for plasma (bottom broken line). After incubation for three minutes, the serum-TP mixture produced no coagulation of fibrinogen as compared to a coagulation time of 28 seconds for the plasma.

A preparation of purified prothrombin² was then added to the fibrinogen solution as follows: Prothrombin was dissolved in 0.85 per cent sodium chloride solution, adjusted to pH 7.5. Aliquots of this solution, representing a known amount of prothrombin, were then added to the fibrinogen solution. Serum activated by thromboplastin was then added to the different solutions of fibrinogen and prothrombin and the coagulation times were recorded. It will be observed from Figure 2 that the reaction curves produced by adding prothrombin to fibrinogen approximate the same type of curve that is obtained when normal plasma is used.

Plasma was prepared to contain varying amounts of prothrombin by the following method: Fresh plasma was freed of prothrombin by adding it to powdered BaCO₃ (method of Fantl and Nance [4]). The plasma and BaCO₃ were shaken vigorously and allowed to react for 20 minutes. The plasma was then freed of BaCO₃ by rapid centrifugation. The supernatant, clear plasma was removed and tested for prothrombin activity by adding thromboplastin and 0.02 M CaCl₂. Complete removal of prothrombin was evident since the adsorbed plasma failed to coagulate. Sufficient

fresh plasma was then added to aliquots of the BaCO₃-treated plasma to produce a 10 per cent, 20 per cent, 30 per cent, and 50 per cent prothrombin concentration in the plasma utilized in experiments presented in Figure 3. These artificially prepared plasmas were studied in the same manner as fresh plasma to determine the effect of adding a serum-TP mixture. It will be observed that decreasing concentrations of prothrombin in plasma were associated with progressively prolonged coagulation times when the serum-TP mixture was added. This was particularly apparent after the serum-TP had been incubated for seven or 15 minutes. Ten per cent and 20 per cent plasma produced no coagulation after addition of the serum-TP mixture incubated for 15 minutes, whereas 100 per cent plasma was coagulated in 64 seconds by the same mixture. The bottom broken-line curve was obtained by activating serum obtained from a patient treated with dicumarol and then adding this serum-TP mixture to plasma containing 10 per cent prothrombin. The significance of this effect will be discussed later.

On the basis of the above experimental data it is felt that there is sufficient evidence to state that serum can be activated by thromboplastin to produce a "prothrombin-converting substance" which is then capable of causing conversion of prothrombin to thrombin in the virtual absence of ionized calcium. Fibrinogen cannot be converted to fibrin directly by the serum-activated substance. The minimal fibrin formation that does occur when serum-TP mixture is added to fibrinogen is probably related to small amounts of prothrombin contaminating the fibrinogen solution or to small quantities of thrombin or prothrombin residues contaminating the activated serum-TP mixture.

Activation of serum obtained from dicumarol-treated patients: It was found that serum from normal individuals, activated by thromboplastin and incubated as previously described, clotted plasma in a characteristic manner. Most sera from patients with various diseases showed little difference in this respect. In the presence of severe infections, however, a somewhat more flattened reaction curve was produced following thromboplastin activation. For example, the coagulation time of plasma after addition of a serum-TP mixture incubated for 15 minutes was 35 seconds in a typical case as compared with 55 seconds for nor-

² The preparations of fibrinogen and purified prothrombin used in these experiments was supplied through the generosity of Dr. W. H. Seegers, for which grateful acknowledgement is made.

mal serum. In these instances it was found that the plasma prothrombin concentration was diminished (Quick method [10]), and in the light of these observations a study was made of sera obtained from patients who were being treated with dicumarol.

The markedly changed pattern of reaction which follows activation of sera by thromboplastin from prothrombin-deficient patients, related to administration of dicumarol, is recorded in Figure 5. Serum D was obtained 12 hours after administration of 300 mg. of dicumarol *per os*, whereas sera C, B, and A, were obtained 36, 60 and 72 hours later. An analysis of the corresponding plasma revealed a reduction of prothrombin concentration from 100 per cent of normal to 33 per cent of normal (Quick's method).

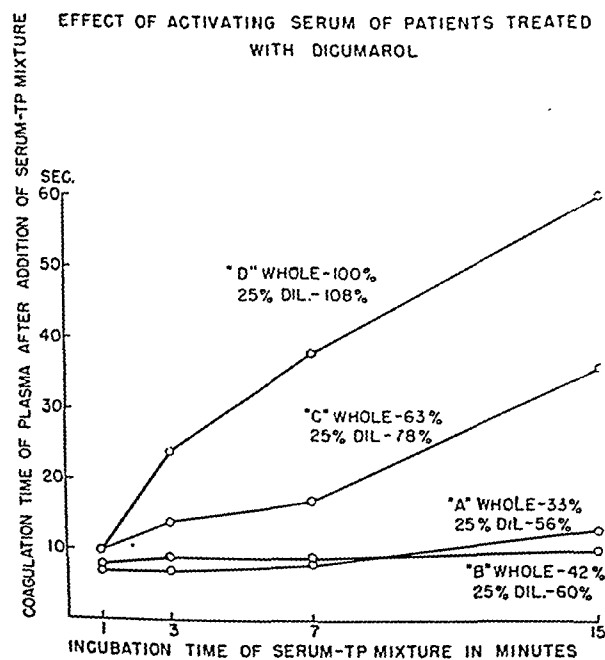


FIG. 5

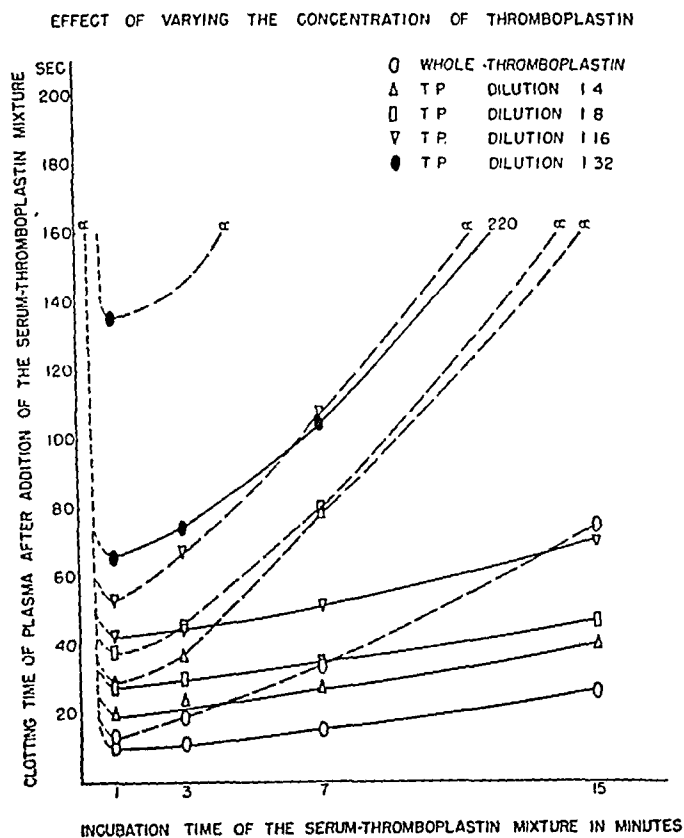
Sera of patients receiving dicumarol were obtained at varying intervals following administration of 300 mg. of dicumarol. Serum D, whose corresponding plasma prothrombin concentration was 100 per cent of normal, was obtained 12 hours after dicumarol was given. Similarly, serum C (plasma prothrombin 63 per cent of normal) was obtained 36 hours after dicumarol; serum B (plasma prothrombin 42 per cent of normal) and serum A (plasma prothrombin 33 per cent of normal) were obtained 60 and 72 hours after dicumarol *per os*. The activated serum-thromboplastin mixture appears to have a more prolonged action as the corresponding plasma prothrombin concentration falls.

It will be observed that the coagulation time of the serum-TP mixture, added to plasma after incubation for 15 minutes, is 10 seconds for serum B, in contrast to 60 seconds for serum D. Similarly, serum C, with an intermediate reduction of the corresponding plasma prothrombin concentration also showed an intermediate reduction of the plasma-serum-TP coagulation time (36 seconds) at the end of the 15 minute incubation period.

When such serial studies are made of patients who are receiving dicumarol, it is possible to show that there exists a fairly good correlation between the shape of the serum-activation curve and the concentration of prothrombin in the plasma. As the concentration of plasma prothrombin falls, a progressive shortening of the coagulation time of the serum-TP mixture develops when this is tested for coagulating effect on normal plasma after incubation for 15 minutes.

Figure 3, to which reference has previously been made, illustrates in another manner the enhanced effect obtained when serum from dicumarol-treated patients is activated by thromboplastin. It will be observed that coagulation of plasma containing 10 per cent concentration of prothrombin was more rapid, following addition of activated serum from a dicumarol-treated patient, than was coagulation of plasma containing 100 per cent prothrombin following addition of activated normal serum.

Serum from a dicumarol-treated patient was more susceptible of activation when smaller amounts of thromboplastin were utilized. This is clearly shown in Figure 6, where varying concentrations of thromboplastin were employed in activation of the normal serum and serum from dicumarol-treated patients. In this experiment 0.1 cc. of undiluted thromboplastin is designated "whole thromboplastin." Twofold dilutions of thromboplastin suspension were then made in 0.85 per cent sodium chloride solution and 0.1 cc. aliquots of the diluted suspensions were then added to 0.5 cc. amounts of serum to effect activation. The activation curves were then determined in the same manner as described in "methods." In Figure 6, the unbroken lines show results obtained when serum obtained from a dicumarol-treated patient was utilized. The plasma prothrombin concentration in this instance was 20 per cent of normal.



Effect of varying the concentration of thromboplastin added to serum to activate the coagulation factor. Respective dilutions of thromboplastin are indicated on the graph. One tenth cc. portions of these dilutions were added to serum. After the designated incubation periods (abscissa), 0.1 cc. amounts of this mixture were added to fresh oxalated plasma. There is a lessened coagulating effect when the amount of thromboplastin is decreased. The difference between activated serum from a patient treated with dicumarol (solid line) and normal serum (broken line) is again demonstrated.

The broken-line curves represent the results obtained after activation of serum from a normal individual whose plasma prothrombin concentration was 100 per cent of normal. These data show that serum obtained from the dicumarol-treated patient was much more effectively activated by thromboplastin than the sera of the normal individual.

It is evident that a striking difference exists between sera from fully dicumarolized patients and normal serum, following activation of these sera by thromboplastin. In fact, the data suggest that a converse relationship may exist between concentration of plasma prothrombin and the concentration of "prothrombin-converting factor" which can be activated from the corresponding serum.

As the plasma prothrombin concentration falls either more "prothrombin-converting factor" can be activated from the serum or the effect of the "prothrombin-converting factor" is more prolonged.

In view of the results obtained in a study of sera from patients treated with dicumarol, the results obtained in a study of a patient with severe cirrhosis of the liver associated with a prothrombin deficiency, are of particular interest. This patient with cirrhosis was found to have a plasma prothrombin concentration 40 per cent of normal (Quick's method [10]) associated with a coagulation time of 16 minutes (Lee-White method [11]). Vitamin K, administered parenterally, produced no change in the plasma prothrombin concentration. No disturbance of blood platelets or of any other coagulation factor was observed. Despite this, the patient developed severe epistaxis, purpuric skin lesions and at exitus he developed hemorrhage into the gastro-intestinal track. This patient's serum was studied on several occasions according to the method described. It showed a decreased ability to coagulate normal plasma after activation of the serum by thromboplastin as contrasted to normal serum. When serum from the patient with hepatic disease was compared with serum obtained from a patient receiving dicumarol with a reduction of prothrombin to a corresponding level, the difference in the two curves was quite evident. Several other patients with cirrhosis of the liver associated with decreased prothrombin have been studied in this manner. None of these patients had any hemorrhagic phenomena or disturbance of whole blood coagulation. In these patients, no results approximated those seen in sera of dicumarol-treated patients which had corresponding reductions of prothrombin. However, none showed any striking deviation of the reaction curve from that obtained following activation of normal serum and none showed such a striking deviation as the above patient with the prothrombin deficiency associated with disturbance of whole blood coagulation.

It is probable that the patient with hepatic disease associated with purpura, et cetera, had a deficiency of "prothrombin-converting factor" as well as prothrombin itself and that both defects may have contributed to the production of the ob-

served abnormality of coagulation and the purpuric manifestations.

Effect of addition of potassium oxalate to serum before and after thromboplastin activation: The preceding experiments have indicated that once activation of the "prothrombin-converting factor" has been accomplished, the reaction of prothrombin conversion can take place under conditions where the amount of ionized calcium is considerably reduced. When the concentration of oxalate in plasma is 0.01 M, coagulation by activated serum proceeds rapidly. If the molar concentration of plasma is increased to 0.02 M, the coagulation effect of activated serum is retarded and finally, no coagulation results if the concentration of oxalate in plasma is 0.04 M.

Furthermore, it was found that activation of serum by thromboplastin followed by addition of potassium oxalate, resulted in a rapid inhibition of coagulation activity of the serum-TP mixture. This loss of coagulation effect was observed even when the serum-TP mixture to which oxalate was added one minute later, was reacted with partially recalcified plasma. This plasma contained sufficient calcium more than to neutralize the amount of potassium oxalate added to the serum-TP mixture. It appears, therefore, that potassium oxalate

is able to reverse the active "prothrombin-converting factor" to an inactive form. This may represent the mechanism by which activated serum-TP mixtures are apparently inhibited in plasma containing high concentrations of potassium oxalate.

Table I demonstrates the effect obtained when potassium oxalate was added to serum *prior* to activation by thromboplastin. One-half cc. aliquots of normal human serum were added to 0.1 cc. amounts of a solution which contained varying concentrations of potassium oxalate. Several minutes later, 0.1 cc. of a thromboplastin suspension was added to the serum-oxalate mixture. Exactly one minute later, 0.1 cc. of the serum-TP-oxalate mixture was added to 0.1 cc. of 0.01 M oxalated plasma. It will be observed that coagulation of plasma resulted as long as the molar concentration of potassium oxalate was less than 0.0036 M. When the concentration of serum oxalate exceeded 0.0037 M, the activation of serum by thromboplastin was inhibited.

In order to eliminate the possibility that a cumulative concentration of potassium oxalate in the combined serum-TP and plasma might influence the speed of coagulation, the following control was made. The same series of serum-TP-oxalate mixtures was added to .005 M plasma. This plasma was prepared by adding 0.05 cc. of 0.1 M calcium chloride to 0.95 cc. of 0.01 M oxalated plasma. The amount of oxalate added to serum was more than neutralized by the concentration of calcium in the 0.005 M plasma. At the same time, plasma prepared in this manner did not spontaneously coagulate. It will be observed (Table I) that the coagulation effect of the serum-TP-oxalate mixture was not enhanced by use of partially recalcified plasma. In fact, the speed of coagulation was prolonged over that obtained in plasma which contained a higher concentration of potassium oxalate. The discrepancy of results obtained by use of 0.01 M and 0.005 M oxalated plasma is not clear, but it seems unlikely that any of the coagulation effect can be related to a direct action of thromboplastin *per se* on the plasma prothrombin.

These data are supportive evidence that prothrombin conversion can occur when the ionized calcium concentration is very low. The importance of calcium in coagulation seems to be re-

TABLE I

Effect of adding potassium oxalate to serum prior to activation by thromboplastin

Final molar concentration of oxalate in the serum*	Coagulation time (sec.) of serum-TP-oxalate mixture added to 0.01 M oxalated plasma	Coagulation time (sec.) of serum-TP-oxalate mixture added to 0.005 M oxalated plasma†
0	26.0	28.0
.0014 M	31.0	31.0
.0021 M	31.0	76.0
.0028 M	36.0	70.0
.0035 M	43.0	83.0
.0037 M	229.0	>300.0
.004 M	>300.0	>300.0

* Concentration obtained by dividing molar concentration of potassium oxalate added by the dilution factor of serum and thromboplastin mixture, *i.e.*,

$$\frac{.02 \text{ M}}{7} \text{ oxalate} = .0028 \text{ M.}$$

† 0.005 M oxalated plasma was made by adding 0.05 cc. of 0.1 M CaCl₂ to 0.95 cc. of 0.01 M oxalated plasma.

Increasing concentrations of oxalate contained in 0.1 cc. were added to 0.5 cc. of serum. Following this, 0.1 cc. portions of thromboplastin suspension were added to the respective sera and one minute later, 0.1 cc. of this mixture was added to 0.1 cc. of 0.01 M or 0.005 M oxalated plasma. The coagulation time was then recorded.

lated to the stage of activation of "prothrombin-converting factor." In addition, once the "prothrombin-converting factor" has been activated, it can be rapidly inactivated if an excess of potassium oxalate is present in plasma or if potassium oxalate is added directly to the active serum-TP mixture.

Effects of physical agents on serum-thromboplastin activation: It has been shown that serum from normal individuals produces a characteristic predictable reaction curve following activation with thromboplastin (Figure 1). There is a release of "prothrombin-converting factor" followed by gradual decay of activity.

A progressive, slow loss of activatable "prothrombin-converting-factor" occurred when the serum was stored at 0° C. prior to addition of thromboplastin. A rapid loss occurred if serum was kept at 37° C., so that 10–12 hours later, only slight activation of the "prothrombin-converting factor" resulted after addition of thromboplastin.

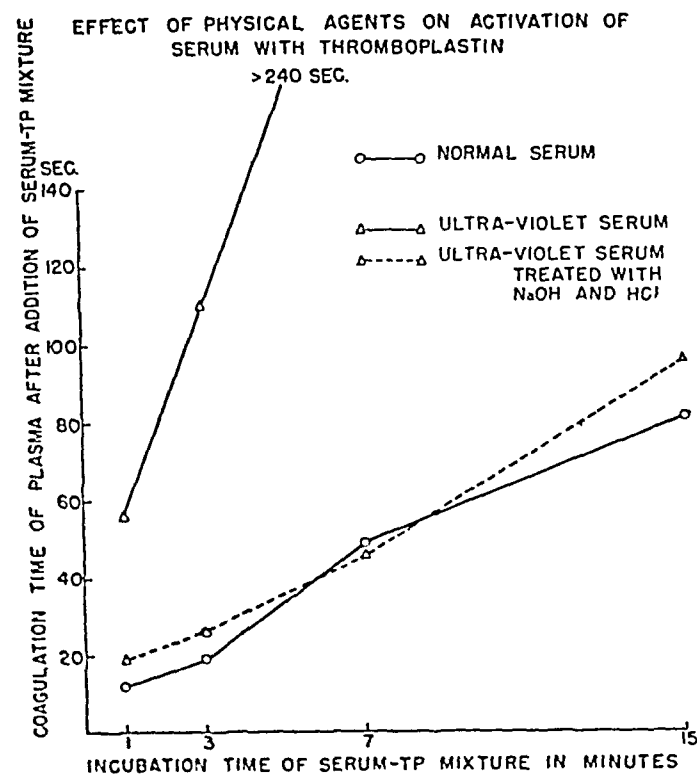


FIG. 7

Effect of change of pH on restoration of activatable serum "prothrombin-converting factor." Ultra-violet irradiation of serum for two hours prior to activation by thromboplastin causes a marked decrease of activatable substance. After this serum was treated with alkali, followed by restoration of pH to 7.3, the activation curve approximates that obtained in the untreated serum.

When serum was irradiated by ultra-violet light, a rapid decrease of the "prothrombin-converting factor," capable of activation by thromboplastin, resulted.

Serum which becomes unreactive by aging or by ultra-violet irradiation can be almost completely restored to activity by treatment with dilute alkali or acid. In experiments illustrated in Figure 7, 0.25 cc. of 0.1 N NaOH was added to 0.5 cc. of inactive serum. Thirty minutes later 0.25 cc. of 0.1 N HCl was added to this mixture to restore the pH to a value between 7.0 and 8.0. When serum was treated in this manner, it was found to have nearly complete restoration of prothrombin-converting activity following addition of thromboplastin.

Storage of serum for as long as one year at refrigerator temperatures does not result in complete loss of the "prothrombin-converting factor." In fact, some of the sera which became alkaline (pH 8.5) after storage, were found to be capable of activation by thromboplastin without preliminary treatment with alkali or acid.

These data indicate that the "prothrombin-converting factor" of serum does not readily deteriorate, but only becomes reversibly inactivated.

Effect of crystalline soybean trypsin-inhibitor on activation of serum by thromboplastin: Since crystalline trypsin-inhibitor substances have been shown capable of delaying blood coagulation by inhibiting prothrombin conversion (12–14) a preparation of soybean trypsin-inhibitor³ was employed to study the effect of this substance by means of this experimental method. The preparation used in this protocol (Figure 8) produced appreciable prolongation of the plasma prothrombin time when 0.1 mg. was added to 0.1 cc. of plasma. Crystalline soybean trypsin-inhibitor was added in varying amounts to two different sera, one of which was obtained from a normal individual, while the other was obtained from a patient receiving dicumarol. The plasma, in the latter instance, was found to have a prothrombin concentration 28 per cent of normal. Examination of Figure 8 reveals that inhibition of activation of serum by thromboplastin was proportional to the amount of trypsin-inhibitor added to the serum. However, the de-

³ Crystalline soybean trypsin-inhibitor was supplied through the generosity of Armour and Company, Chemical Research Division, Chicago 9, Illinois.

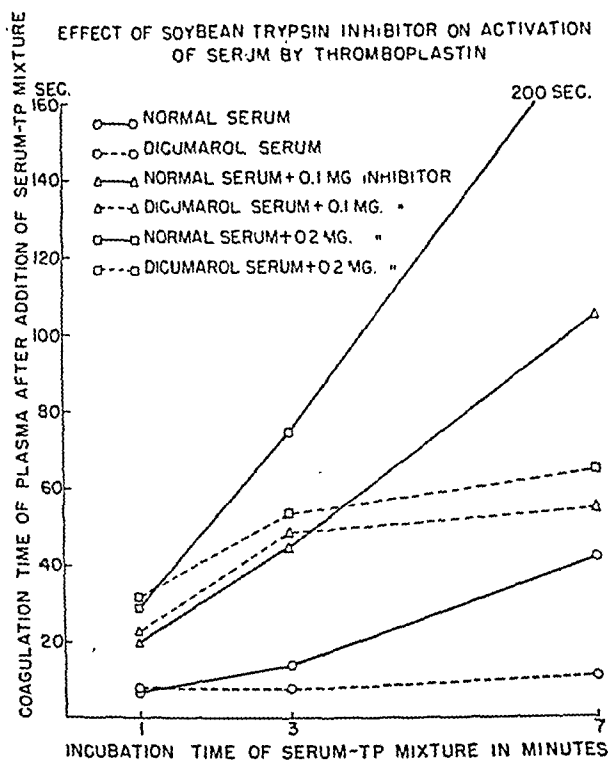


FIG. 8

Soybean trypsin-inhibitor was added to serum (amount designated on the graph) prior to activation by thromboplastin. Two types of sera are shown, the solid lines representing normal sera, whereas the broken lines represent sera obtained from a dicumarol-treated patient. Trypsin-inhibitor reduces the activity of "prothrombin-converting factor," particularly after the serum-thromboplastin mixture with trypsin-inhibitor added has been incubated for seven minutes. In contrast, the "dicumarol serum" was less affected by the trypsin-inhibitor.

curve of the "prothrombin-converting factor" was affected less in the dicumarol than in the normal serum. For example, the serum-TP mixture, to which 0.2 mg. of trypsin-inhibitor had been added, was incubated for seven minutes and was found to produce coagulation of plasma in 200 seconds when normal serum was employed, in contrast to a coagulation time of plasma of 65 seconds when serum obtained from the patient treated with dicumarol was used.

Crystalline soybean trypsin-inhibitor was added to plasma in sufficient concentration to reproduce the amount which would be carried over in the above serum-TP mixtures. Serum was then activated by thromboplastin in the usual manner and added to the plasma-trypsin-inhibitor system. It was found that no inhibition of the activated "pro-

thrombin-converting factor" resulted. This observation indicates that soybean trypsin-inhibitor delays coagulation either by inhibition of thromboplastin or by inhibition of activation of the serum "prothrombin-converting factor."

DISCUSSION

When plasma coagulates, it is apparent that several constituents are either removed or destroyed as a result of the coagulation process. Prothrombin is removed by being converted to thrombin, which in turn is either destroyed or combined with other plasma components to form "metathrombin" (10). Fibrinogen is converted to fibrin which can then be removed as a clotted mesh. Serum, another end product of coagulation, contains, therefore, only small amounts of prothrombin and thrombin which deteriorate on standing and "metathrombin" which has been reported capable of reconversion to thrombin under suitable experimental conditions (15). At first it seemed logical to suppose that the coagulating effect of serum to which thromboplastin had been added was related to reconversion of "metathrombin" to thrombin. However, from the results of experiments with fibrinogen solutions and deprothrombinated plasmas it became apparent that the coagulating effect of activated serum could not be due to thrombin, but rather represented some factor which initiated coagulation through an intermediate action on prothrombin. It therefore seemed necessary to conclude that serum contains a "prothrombin-converting factor" which can be activated by thromboplastin. During the process of blood coagulation, the "prothrombin-converting factor" present in an inactive state *in vivo* in circulating plasma is activated either by thromboplastic globulin or tissue, or platelet thromboplastin to produce an active "prothrombin-converting factor" which in turn causes conversion of prothrombin to thrombin. Following the initial release of "prothrombin-converting factor" it is then converted to an inactive substance producing no effect on coagulation until more thromboplastin is added to reactivate it.

When serum is utilized to study the activation of the "prothrombin-converting factor" there is no substrate upon which this factor can effect a normal reactive process. It has been possible, therefore, to dissect the first stage of blood coagulation

and study this process more completely. By this method of study one can demonstrate a sudden release of "prothrombin-converting factor" soon after serum has been in contact with a suspension of thromboplastin. Following this, the "prothrombin-converting factor" slowly disappears from the serum-thromboplastin mixture. The degree of release of "prothrombin-converting factor" is directly related to the concentration of thromboplastin added to the serum for activation. Similarly, if one uses plasma which contains a reduced concentration of prothrombin, the clotting time will be correspondingly prolonged following addition of an active serum-TP mixture.

The mechanism by which the inactive serum "prothrombin-converting factor" is activated by thromboplastin is a topic for critical consideration. Ferguson (12), MacFarlane and Pilling (13), and Grob (14) have shown that serum and pancreatic trypsin-inhibitor substances have an appreciable influence on *in vitro* prothrombin conversion. It has been shown above that a crystalline soybean trypsin-inhibitor substance can appreciably alter the reaction curve when this is added to serum which is then activated by thromboplastin. Whether this effect is related to a neutralization of thromboplastin as MacFarlane and Pilling (13) believe, thereby diminishing the activating effect of the thromboplastin for serum, is not clear.

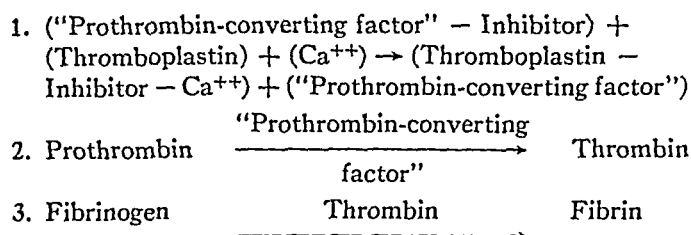
The type of reaction seen in the serum which has been aged or treated with ultra-violet irradiation may be related to a reversible denaturation of the reacting system, so that thromboplastin can no longer activate the "prothrombin-converting factor." When serum is treated by changing the pH, as described, then the "prothrombin-converting factor" again becomes capable of activation by thromboplastin in much the same manner as seen in fresh, normal serum.

On the basis of studies made on serum obtained from patients treated with dicumarol, it is apparent that there is a decided change produced in plasma, irrespective of the effect of dicumarol on the plasma prothrombin concentration. The data presented suggest that dicumarol administration is associated not only with a decreased concentration of prothrombin in the plasma, but also an increase of "prothrombin-converting factor." If this type of balance operates to preserve homeostasis of the coagulative mechanism, in that a reduction of pro-

thrombin would be counter-balanced by an increased concentration of "prothrombin-converting factor," then the enigma of marked prothrombin reduction without a profound disturbance of the coagulation mechanism might be more easily comprehended. A different and adverse situation appeared to be present in the patient with cirrhosis of the liver, who had a moderate reduction of prothrombin with associated hemorrhagic and coagulative disturbance, and at the same time was found to have a decrease in "prothrombin-converting factor."

One must consider furthermore that the altered reactivity of activated serum from dicumarol-treated patients might be associated with a decrease of inhibitor substance, thereby permitting a prolonged effect of the "prothrombin-converting factor."

On the basis of the experimental evidence presented, a revised formula of blood coagulation is suggested.



The validity of this equation might be strengthened if it could be determined whether thromboplastin is altered after it is added to serum. It has already been pointed out that thromboplastin remains unchanged if one attempts to determine the activity of thromboplastin by *in vitro* coagulation studies.

In vivo studies by Thomas (16) indicate that thromboplastin is changed when it is incubated with serum. He observed that thromboplastin injected intravenously into mice produced death by causing intra-vascular clotting. If thromboplastin was allowed to react with rabbit serum for several hours, many times a minimal lethal dose of thromboplastin could be injected intravenously without producing any adverse effect on the mice. Despite this, the "inactivated" thromboplastin had normal coagulative properties when *in vitro* studies were made. Thomas (16) treated thromboplastin with serum, following which he centrifuged the thromboplastin suspension free of the serum. When this

preparation was treated with potassium oxalate, it again became reactive to produce intra-vascular coagulation of blood. Similarly, he showed that thromboplastin was not inactivated by adding it to oxalated plasma and would continue to produce death in mice following intra-venous injection. These observations suggested to Thomas that a thromboplastin-inhibitory component of serum combined with thromboplastin in the presence of ionized calcium. This combination was easily dissociable if calcium ion was removed.

If these observations are applied to the equation suggested above to explain blood coagulation, it appears that the mode of activation of the "prothrombin-converting factor" might be related to a combination of thromboplastin with an inhibitor to release the "prothrombin-converting factor." It has been shown that such activation cannot take place unless ionized calcium is present.

It is much more difficult to explain how the serum-TP mixture finally becomes inert, only to be reactivated again by adding more thromboplastin or by adding more fresh serum.

No attempt has been made to correlate the above described "prothrombin-converting factor" with the chloroform-activated factor of serum and plasma, described by Tagnon *et al.* (17, 18). These workers have shown that treatment of serum or plasma with chloroform produces a substance which causes conversion of prothrombin to thrombin in the absence of ionized calcium. In addition, this factor causes lysis of fibrinogen and fibrin. When plasma was coagulated by a serum-TP mixture, according to the method described in this report, no lytic action of the serum-TP mixture was observed. However, Tagnon and co-workers have shown that thromboplastin and prothrombin inhibit the lytic action of the chloroform-activated factor.

It is possible that the chloroform-activated factor may be identical with the thromboplastin-activated factor described in the above experiments. Chloroform may cause a selective destruction of the inhibitor in the postulated "prothrombin-converting factor"-inhibitor complex, thereby permanently releasing a "prothrombin-converting factor."

SUMMARY

1. Preliminary investigations are reported which describe a method of activation of a pro-

thrombin-converting substance ("prothrombin-converting factor") by adding thromboplastin to serum. The activated "prothrombin-converting factor" causes conversion of prothrombin in the virtual absence of calcium; slowly decreases in activity following liberation from serum by thromboplastin; can be reactivated again by adding more thromboplastin to the inactive serum-thromboplastin mixture; and is unable to cause direct conversion of fibrinogen to fibrin.

2. Serum of dicumarol-treated patients, which is activated by thromboplastin, has a more sustained release of "prothrombin-converting factor." The activity of "prothrombin-converting factor," under these circumstances, increases as the level of plasma prothrombin falls. Conversely, a patient with cirrhosis of the liver, associated with an hemorrhagic disorder, was found to have a decrease of "prothrombin-converting factor" as well as a reduction of plasma prothrombin.

3. On the basis of the experimental evidence outlined, a revision of the generally accepted theory of blood coagulation is proposed.⁴

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STUDIES IN IRON TRANSPORTATION AND METABOLISM. VII. EVIDENCE OBTAINED WITH RADIOACTIVE IRON THAT "EASILY SPLIT-OFF" BLOOD IRON IS AN ARTEFACT¹

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Three different forms of blood iron have been described: hemoglobin iron, plasma (transport) iron, and "easily split-off" iron (E.S.Fe). The latter fraction, first studied by Barkan (1) and Lintzel (2), constitutes 5 to 10 per cent of the total blood iron. It derives its name from the fact that when blood is acidified and incubated with dilute hydrochloric acid the "easily split-off" fraction is ionized so that it can be separated from the remaining hemoglobin iron by dialysis or ultrafiltration. Lintzel and Radeff (3), and Legge and Lemberg (4) have demonstrated that E.S.Fe may be obtained in a similar manner from crystalline hemoglobin.

The amount of iron which can be "easily split off" from hemoglobin can be influenced in several ways. If blood or hemoglobin is treated with carbon monoxide, with reducing substances (*i.e.*, ascorbic acid, $\text{Na}_2\text{S}_2\text{O}_4$), or is evacuated of its oxygen prior to the addition of dilute hydrochloric acid, the ionization produced by the acid is reduced considerably (4-6). In addition, changes in the concentration of acid alter the size of the fraction (7-9). It is largest when the concentration of HCl is approximately 0.03 N and decreases progressively as the amount of acid is increased to about 0.6 N; further increases in HCl then cause comparatively little additional change. At concentrations of 0.1 N, nitric and sulfuric acids ionize nearly twice as much of the iron in red cells as does hydrochloric acid (7).

The nature and biologic significance of E.S.Fe blood iron have remained in doubt. Some workers have regarded the fraction as an artefact produced by the oxidation of the prosthetic group of the hemoglobin molecule by oxygen evolved when

acids are added to oxyhemoglobin. Barkan and Schales, however, presented evidence to show that E.S.Fe is derived from an intracorpuseular degradation product of hemoglobin, a bile pigment-iron-globin compound with an open porphyrin ring which they called pseudohemoglobin (6, 10).² This explanation was altered by Legge and Lemberg (4). After confirming the observation (6, 10), that about two-thirds of the E.S.Fe fraction was split-off only in the presence of oxygen and in the absence of reducing substances while the remaining third was ionized even in inert gases or CO, they concluded that the larger of these two fractions is an artefact while the smaller is at least partially attributable to a bile pigment-hemoglobin which gives rise to bile pigments when treated with acids. Even though these interpretations differ, both groups of investigators believe that at least a portion of E.S.Fe is derived from an intraerythrocytic pigment formed as one of the initial steps in the breakdown of hemoglobin to bilirubin.

On the other hand, the experiments with radioactive iron reported by Miller and Hahn (13) suggested that E.S.Fe is really an artefact. These observers administered radioactive iron to dogs and observed that several days later there was as much of the isotope present in the E.S.Fe of red blood cells as was found two weeks later. If all of the E.S.Fe is derived from a degradation pro-

² Pseudohemoglobin was prepared *in vitro* by Barkan and Schales by oxidizing oxyhemoglobin with hydrogen peroxide in the presence of cyanide (10). Legge and Lemberg (4), in many of their studies, used choleglobin prepared by coupled oxidation of hemoglobin and ascorbic acid (11). In both compounds, the $\alpha\text{-CH=}$ bridge of the heme molecule is open, and iron is readily dissociated by dilute acids. Both may be classified as verdohemoglobins. Liébecq regards them as being very similar, if not identical substances (12).

¹ These studies were supported by research grants established in memory of Philipp Hunkel and Nathan Greenberg.

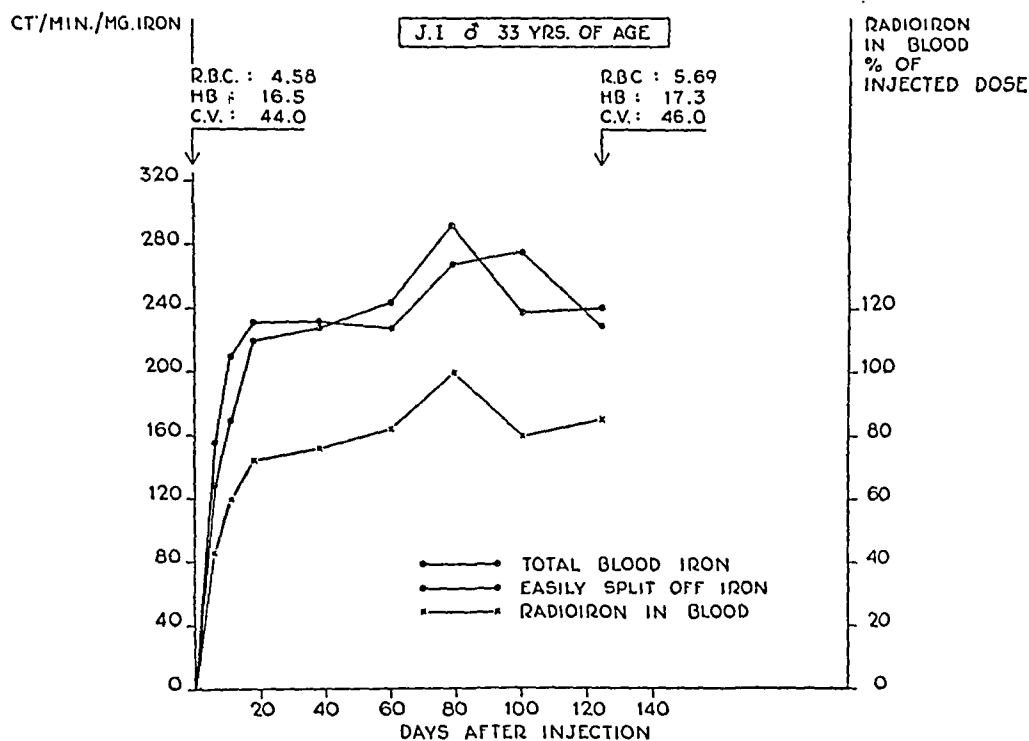


FIG. 2. CONCENTRATION OF RADIOIRON APPEARING IN WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON OF A HEALTHY ADULT MAN AFTER INTRAVENOUS INJECTION OF Fe^{59}

5.6 mgm. of radioiron (Fe^{59}) having 7,200,000 counts per minute were given intravenously as ferrous ascorbate.

(See also explanations for Figure 1.)

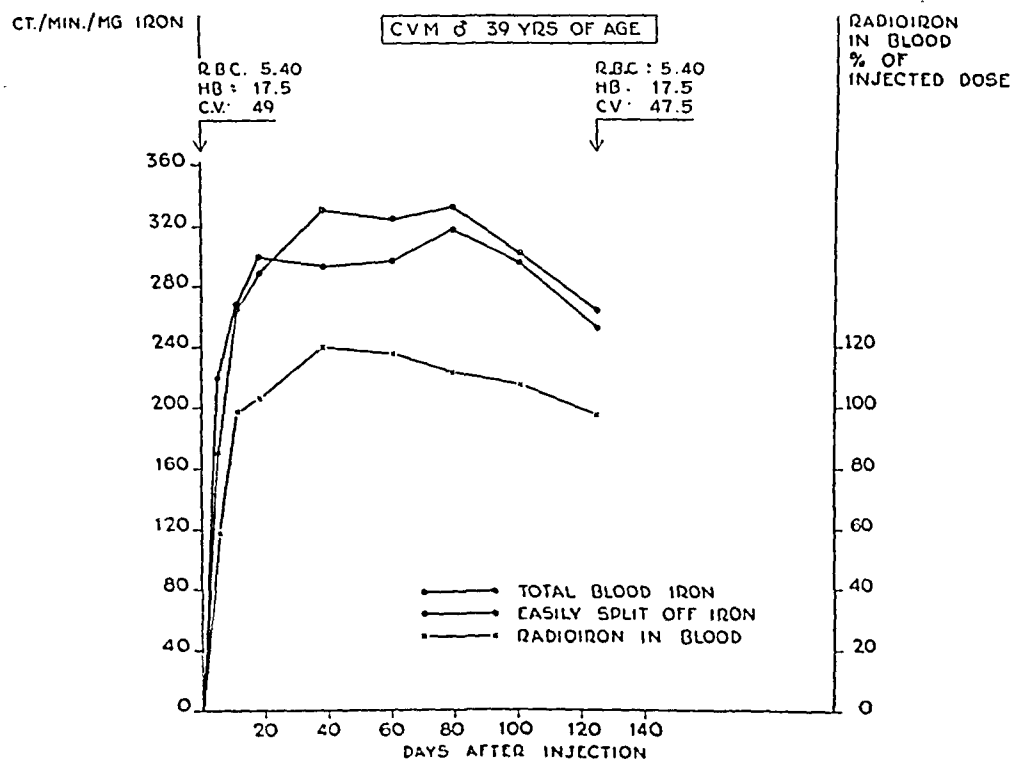


FIG. 3. CONCENTRATION OF RADIOIRON APPEARING IN WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON OF A HEALTHY ADULT MAN AFTER INTRAVENOUS INJECTION OF Fe^{59}

5.6 mgm. of radioiron (Fe^{59}) having 7,200,000 counts per minute were given intravenously as ferrous ascorbate.

(See also explanations for Figure 1.)

TABLE I
Comparison of radioactivity in whole blood iron, "easily split-off" iron separated from oxyhemoglobin, and "easily split-off" iron separated from blood which had been saturated with carbon monoxide

Subject	Radioactive Fe ⁵⁹ given I.V. as ferrous ascorbate	Days after injection of radio-iron	Total blood Fe			"Easily split-off" blood Fe (from Oxy Hb)			"Easily split-off" blood Fe (from CO Hb)		
			Radioactivity			Radioactivity			Radioactivity		
			mgm./100 ml. blood	ct./min./100 ml. blood	ct./min./mgm. Fe	mgm./100 ml. blood	ct./min./100 ml. blood	ct./min./mgm. Fe	mgm./100 ml. blood	ct./min./100 ml. blood	ct./min./mgm. Fe
M. G.—normal, ♂	total counts/min.	124	58.1	14,610	252	2.40	565	236	0.72	136	189
J. I.—normal, ♂	5.6	124	59.6	14,350	240	2.26	513	228	0.51	107	210
C. V. M.—normal, ♂	5.6	124	58.0	15,000	259	2.19	554	253	0.68	185	273
J. M.—pernicious anemia responding to B ₁₂ , ♀	8.6	32	43.2	38,120	882	2.09	1400	670	1.35	855	634
F. M.—malignant hypertension, ♂	22.5*	48	41.7	34,400	824	2.21	1161	540	1.93	1260	654
		7	25.6	58,650	2291	0.91	2070	2275	0.46	993	2100
		2†				0.91	2165	2380	0.44	952	2162
		20†				0.97	2090	2155	0.73	1532	2130
		28†				1.09	2510	2300	0.74	1728	2335

* One-half the amount given on successive days.
† Days blood was kept refrigerated at 4° C. before determinations were made.

In neither instance was there any significant difference between the radioactivity per milligram of T.B.Fe and that per milligram of E.S.Fe. The greatest discrepancies appeared on days 24, 41, and 62 in Dog I. The significant fact, however, is that there was no tendency for the radioactivity in the E.S.Fe fraction to increase as the red cells containing the isotope grew older. In the legends to Figures 4 and 5 are tabulated representative blood counts obtained during the course of the experiment. From these data it is evident that the hemoglobin increased in each animal as it recovered from the anemia induced by hemorrhage. This increase in hemoglobin caused a dilution of the radioiron in the blood and accounts for the gradual decrease in the radioactivity per milligram Fe in both the T.B.Fe and E.S.Fe.

In Dog III, a hemorrhagic anemia was also induced. This animal was followed for only 22 days after he had been given radioiron orally; E.S.Fe was determined after blood had been saturated with CO. The counts per minute per milligram of iron in the T.B.Fe fraction agreed very well with the activity in the fraction separated after exposure to CO (Table II).

TABLE II
Comparison between the concentration of radioactive iron in whole blood and in the "easily split-off" blood iron fraction, determined after saturation with carbon monoxide
Dog III

Days after oral administration	Total blood Fe			"Easily split-off" Fe determined after saturation with carbon monoxide		
	Radioactivity			Radioactivity		
	mgm./100 ml. blood	ct./min./100 ml. blood	ct./min./mgm. Fe	mgm./100 ml. blood	ct./min./100 ml. blood	ct./min./mgm. Fe
8	42.65	512,500	12,000	0.94	11,370	12,100
22	44.60	524,000	11,750	1.06	12,080	11,400

The animal had been made anemic by repeated bleeding prior to administration of radioiron. 47.6 mgm. of radioactive iron (Fe⁵⁵ and Fe⁵⁹) were given by stomach tube, as ferric chloride. Total counts = 11,900,000/min.

III. Human subject with hypochromic anemia

A study similar to that described in the two previous sections was made on a woman, S. W., 35 years of age, with a severe degree of hypochromic anemia (Figure 6). In this instance, the radioiron was given orally. The absorbed isotope

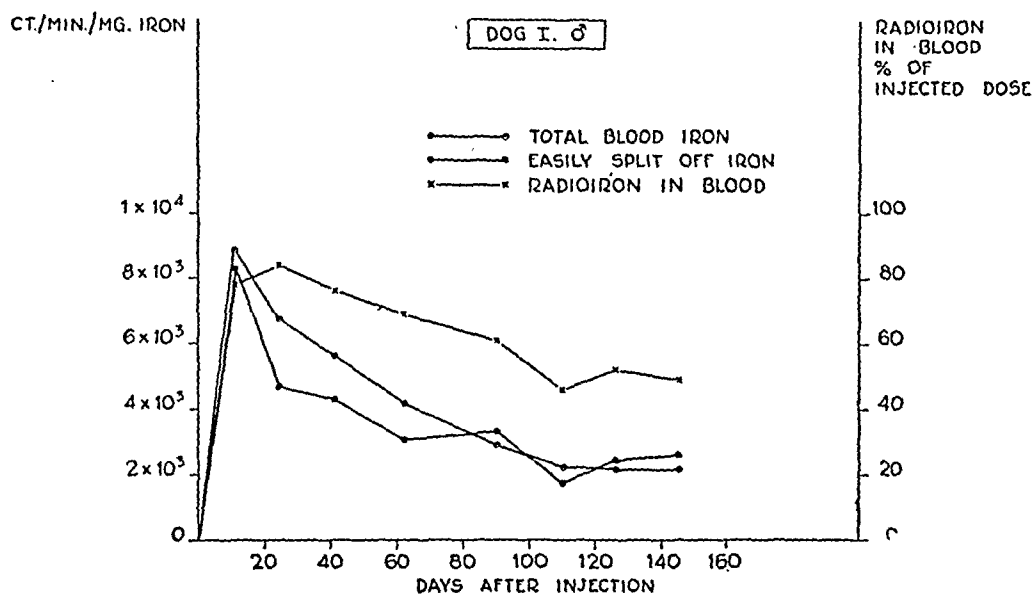


FIG. 4. CONCENTRATION OF RADIOIRON APPEARING IN WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON OF A DOG MADE ANEMIC BY INDUCED HEMORRHAGE

17.4 mgm. of radioiron (mixture of Fe^{55} and Fe^{59}) were given intravenously as ferric chloride. Total counts = 7,200,000/min.

Day	R.B.C.	Hb.	C.V.
1	3.26	6.5	22.5
11	5.48	10.3	35.5
41	6.22	14.4	46.0
90	7.77	15.8	49.0
145	7.20	15.9	49.5

(See also explanations for Figure 1.)

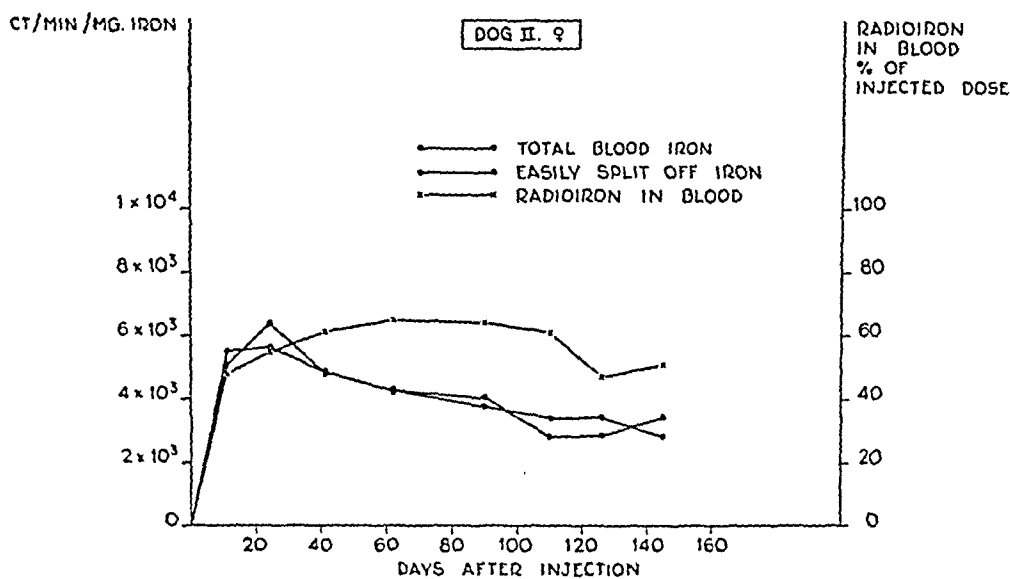


FIG. 5. CONCENTRATION OF RADIOIRON APPEARING IN WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON OF A DOG MADE ANEMIC BY INDUCED HEMORRHAGE

34.8 mgm. of radioiron (mixture of Fe^{55} and Fe^{59}) were given intravenously as ferric chloride. Total counts = 14,400,000/min.

Day	R.B.C.	Hb.	C.V.
1	3.41	6.5	23
11	5.21	10.2	35.5
41	6.8	15.0	46
90	7.35	15.6	47
145	6.9	18.4	50

(See also explanations for Figure 1.)

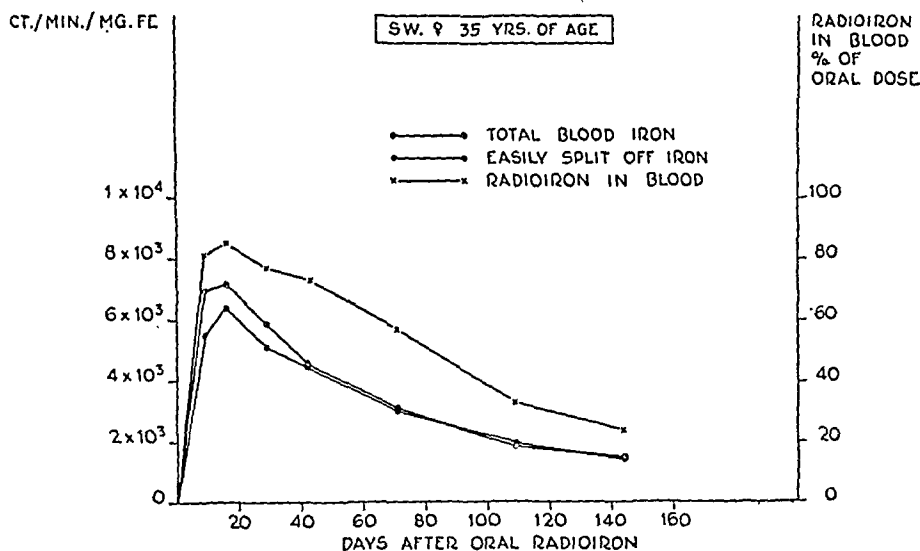


FIG. 6. APPEARANCE OF RADIOIRON IN THE WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON FRACTION OF A PATIENT WITH HYPOCHROMIC ANEMIA

55 mgm. of radioiron (mixture of Fe^{55} and Fe^{59}) given orally as ferrous chloride. Total counts = 32,000,000/min. Patient was treated with 2.4 gm. of ferrous gluconate daily after the 16th day.

Day	R.B.C.	Hb.	C.V.
1	4.41	8.6	28
16	4.11	8.7	27.5
42	5.11	10.1	35
71	4.37	12.4	38
144	3.82	10.1	32

(See also explanations for Figure 1.)

was utilized promptly for hemoglobin synthesis, but by the time observations were discontinued on the 144th day the amount of radioiron in her peripheral blood had decreased to approximately one-third of the peak value. This fall was unquestionably caused by menorrhagia with the loss of large amounts of blood.

As in the normal subjects and in the dogs, the radioactivity per milligram of iron in both T.B.Fe and E.S.Fe remained almost identical during the whole period of study.

IV. Patients with Addisonian pernicious anemia

In order to study the differences which might occur in the radioactivity present in T.B.Fe and E.S.Fe during the first few days after administration of the isotope, it was necessary to select subjects who were manufacturing hemoglobin at an accelerated rate. This precaution was required to make certain that the amount of radioiron pres-

ent in the fractions during the first few days would be large enough to count. Accordingly, radioiron was given intravenously to three patients with Addisonian pernicious anemia during the period of accelerated erythropoiesis following the institution of specific therapy. The results for two of these observations are charted in Figures 7 and 8. Utilization of the injected isotope was prompt and approximately complete. In the first subject (O. A. W., Figure 7) more radioactivity was discovered during the first two days per milligram of iron in the E.S.Fe fraction than in the T.B.Fe. As will be emphasized later, this increase was probably due to the radioactive iron remaining in the plasma. After the first 48 hours, the radioactivity per milligram of iron in E.S.Fe remained slightly but consistently lower than in T.B.Fe.

Determinations of E.S.Fe after blood had been saturated with carbon monoxide were made on the second patient (J. M., Figure 8). This patient was the only one of all the subjects studied in

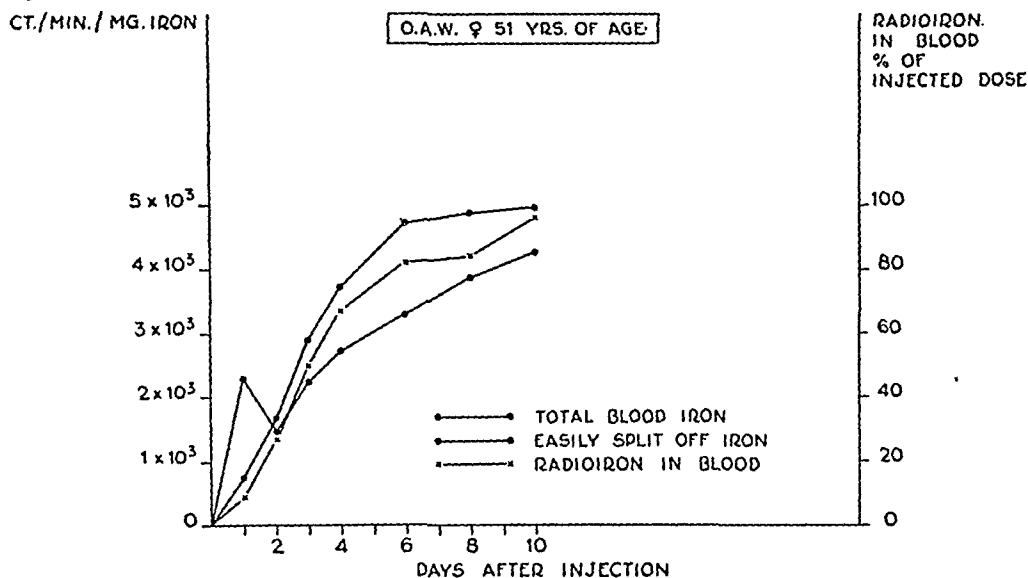


FIG. 7. APPEARANCE OF RADIOIRON IN THE WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON FRACTION OF A PATIENT WITH PERNICIOUS ANEMIA DURING RESPONSE TO LIVER EXTRACT

Liver extract in therapeutic doses had been given approximately two weeks prior to the injection of radioiron. 6.5 mgm. Fe^{59} as ferrous ascorbate injected intravenously. Total counts: 10,980,000.

Day	R.B.C.	Hb.	C.V.
1	2.75	7.8	27
10	3.77	11.3	35.5

(See also explanations for Figure 1.)

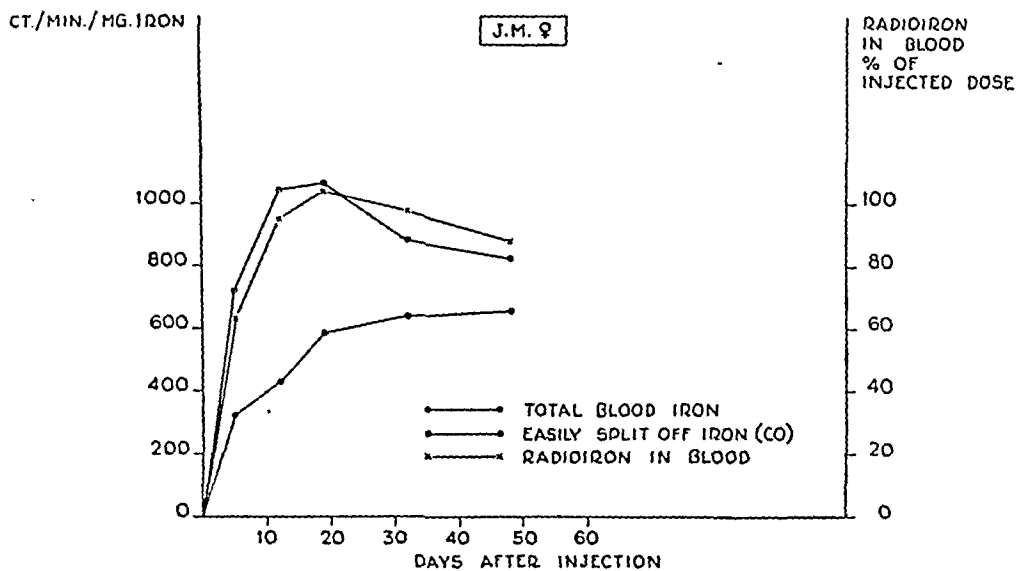


FIG. 8. APPEARANCE OF RADIOIRON IN THE WHOLE BLOOD AND "EASILY SPLIT-OFF" BLOOD IRON FRACTION OF A PATIENT WITH PERNICIOUS ANEMIA DURING RESPONSE TO VITAMIN B_{12}

Three weeks prior to the radioiron 46 μg . of Vitamin B_{12} had been given intramuscularly. Five mgm. of Fe^{59} as ferrous ascorbate injected intravenously. Total counts: 2,840,000. Red blood cell values were increasing rapidly at the time the iron was administered.

(See also explanations for Figure 1.)

whom the radioactivity per milligram Fe of E.S.Fe differed significantly from that in T.B.Fe. It was consistently lower during the 48 days of observation; further study became impossible when the patient left the hospital. The reason for this different result is not clear. That it was not due to the fact that determinations were done in CO was demonstrated on the 32nd and 48th days when E.S.Fe was measured in the usual manner (Table I); a similar lower activity was found.

The radioiron in plasma during the first 48 hours after injection of radioactive ferrous ascorbate was followed in the third subject (W.S., Table III).

TABLE III

Radioactive Fe in the plasma of a patient with pernicious anemia (W. S.) during first 48 hours after intravenous injection of Fe⁵⁹

Time following radioiron I.V. hours	Counts/min./100 ml. plasma
15	6380
24	3120
48	880

Five mgm. Fe⁵⁹ given I.V. as ferrous ascorbate 21 days after initiation of liver therapy. Red blood cell values were rising rapidly during the time these observations were made. Total counts administered = 7,900,000.

It can be seen that relatively large amounts of radioactivity were present in the plasma during this period. Since the plasma iron is also ionized by the concentrations of acid used for determination of E.S.Fe, the radioactivity in this latter fraction would tend to be falsely high during the first several days after injection of radioactive iron. This fact probably accounts for the high concentrations of Fe⁵⁹ in the E.S.Fe fraction observed at 24 and 48 hours in O. A. W. (Figure 7).

V. Effect of storing blood

If hemoglobin transformation to bilirubin begins within the red blood cell by formation of a verdohemoglobin, then it is possible that the intracorpuseular degradation of hemoglobin would take place more rapidly in stored blood. Should this occur in blood which contains hemoglobin tagged with Fe⁵⁹, one would expect that the radioactivity per milligram of iron would progressively increase in the E.S.Fe fraction. To test this possibility, blood obtained from F. M., an adult male with malignant hypertension, was collected on the seventh day following the first of two injections

of tagged ferrous ascorbate. As described in the Methods Section, the blood was stored in small flasks at 4° C. On the second, 20th, and 28th days, flasks were removed for analysis. While there was a tendency for the total amount of iron in the E.S.Fe fraction to increase, particularly when CO was used to saturate the blood, there was no appreciable change in the radioactivity per milligram of iron (Table I). Observations had to be terminated after 28 days because the cells became hemolyzed.

DISCUSSION

In all observations except those made on one of the patients with pernicious anemia (J. M., Figure 8), there was extremely close correlation between the radioactivity per milligram of iron in whole blood and that in the "easily split-off" fraction. Radioiron appeared just as promptly in the E.S.Fe fraction as in the T.B.Fe. There was, furthermore, no tendency for the concentration of radioactivity in the E.S.Fe to increase as erythrocytes aged and reached the end of their life span, as would be expected if E.S.Fe bore any relationship to the formation of an intracorpuseular degradation product of hemoglobin. The same type of result was obtained whether E.S.Fe was determined on oxygenated blood or on blood which had been saturated with carbon monoxide prior to the addition of acid. The data indicate that "easily split-off" blood iron as defined by Barkan is an artefact and originates entirely or almost entirely from intact hemoglobin.

These observations do not, however, eliminate the possibility that a small fraction of E.S.Fe may originate from an intraerythrocytic verdohemoglobin like choleglobin or pseudohemoglobin. It is conceivable that the methods used were not delicate enough to detect the small increase in concentration of radioiron which would occur in E.S.Fe under these circumstances as the cells aged. That such a possibility exists, is suggested by Lemberg's and Legge's (17) demonstration that: (1) normal human red blood cells contain 1 to 5 µg. of biliverdin combined as choleglobin per milliliter of cells; and (2) the injection of phenylhydrazine into rabbits causes an increase in the choleglobin of their erythrocytes. Gardikas, Kench and Wilkinson (18), however, have recently criti-

cized the technique used by Lemberg and Legge on the grounds that choleglobin might well have been formed during their determinations. Gardikas *et al.* repeated the observations under anaerobic conditions and failed to detect choleglobin in 12 separate samples of blood. Furthermore, except in one instance each of leukemia, pernicious anemia, and obstructive jaundice, they failed to demonstrate the pigment in blood taken from patients with pernicious anemia, leukemia, hemolytic anemias, polycythemia, or hepatic and obstructive jaundice. At the present time, therefore, there remains no unchallenged demonstration that any pigment formed as a degradation product of hemoglobin regularly occurs within red blood cells. There is no convincing evidence that the transition of hemoglobin to bilirubin begins as an intraerythrocytic process. It is emphasized that even if such a pigment were found to be present, it would account for only a small fraction of E.S.Fe and would not seriously affect the conclusion that the E.S.Fe fraction is an artefact.

SUMMARY AND CONCLUSIONS

At intervals following the administration of radioactive iron to normal human subjects, to patients with hypochromic and pernicious anemias, and to dogs made anemic by repeated bleeding, the concentration of radioactive iron in the total blood iron and in "easily split-off" iron was measured. In some of the observations "easily split-off" iron was determined on samples of blood which had been saturated with carbon monoxide prior to the addition of acid. In one experiment, citrated blood was stored at 4° C. and determinations were made at intervals for 28 days. Radioactivity appeared in the "easily split-off" blood iron fraction just as promptly and in the same concentration as it appeared in hemoglobin. No correlation was noted between the radioactivity in the "easily split-off" iron fraction and ageing of the red blood cells. It is concluded that "easily split-off" blood iron, whether determined on oxygenated blood or on blood saturated with carbon monoxide, is an artefact. The possibility exists that a small fraction of the "easily split-off" iron may be derived from intraerythrocytic verdohemoglobin, but no convincing evidence exists for the presence in red

cells of such a pigment nor for the belief that degradation of hemoglobin begins within the erythrocyte.

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A COMPARISON OF THE DIRECT FICK AND THE GROLLMAN METHODS FOR DETERMINATION OF THE CARDIAC OUTPUT IN MAN

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The introduction of the cardiac catheter by Forssman (1) and its later development as a useful physiological instrument by Cournand (2, 3) have made possible the determination of the cardiac output by the direct Fick principle in man (4). Using this method, a number of investigators have reported values of 5.5 to 6.5 liters per minute for the basal cardiac output, which correspond to 3.1 to 3.8 liters per minute per square meter of body surface area (3, 5-8). These values are higher than those obtained earlier with the Grollman acetylene inhalation method or the Krogh-Lindhard nitrogen oxide method (3.9 liters per minute) (9-12). The arteriovenous oxygen difference, determined according to the Fick method, has been found to be 40 to 45 cc. per liter of blood, as compared with 60 cc. per liter of blood using the acetylene method. This discrepancy has been discussed at length. It has been suggested by those working with the gas inhalation methods, that the heart catheterization procedure so disturbs the subject, that basal values cannot be secured. Different investigators using the catheter technique have obtained the same values in similar series, without an increase in oxygen consumption, heart rate or blood pressure (3), and have pointed out that these values have all varied consistently from those obtained by the Grollman technique.

The purpose of the work described in this study was to determine the cardiac output simultaneously by both methods and to account for any differences noted.

MATERIAL AND METHOD

Medical students, nurses or patients with minor cardiovascular diseases were used as subjects.

The subjects were trained for the Grollman technique the day before the study. The heart catheterization was done according to earlier descriptions (3, 8). The sample of mixed venous blood was always taken from the

pulmonary artery and the arterial blood from the brachial artery. The first cardiac output was determined according to the Fick principle after the heart catheter and the arterial needle had been in place for at least 30 minutes.

Immediately after the first Fick determination, the acetylene rebreathing was performed. The first sample of alveolar air was taken 17 to 18 seconds and the second 26 to 27 seconds after the start of rebreathing acetylene (10, 11, 13, 14). Twenty to 30 minutes later the same procedure was repeated. Twenty to 30 minutes later a third Grollman determination was done.

Twenty minutes later a single blood sample was drawn from the pulmonary artery. Immediately afterward a rebreathing according to Grollman but without acetylene was performed. Another blood sample from the pulmonary artery was drawn between 17 and 27 seconds after the start of rebreathing. These samples were analyzed for oxygen content on the Van Slyke apparatus in order to establish the influence of the rebreathing procedure on the mixed venous blood and the arteriovenous oxygen difference.

In three subjects the effect of recirculation was studied. Blood was drawn in five second periods from the pulmonary artery, during the rebreathing of acetylene. The blood was analyzed for acetylene content of the Van Slyke volumetric apparatus. In each subject two to three experiments were done. It was usually possible to obtain blood during two five second periods at each experiment, for example, between five to 10 and between 15 to 20 seconds after the start of acetylene breathing.

RESULTS

In Table I the age of the subject, diagnosis, oxygen consumption, and the arteriovenous oxygen difference obtained with the two methods are tabulated. The values for arteriovenous oxygen difference using the acetylene method were always larger than those obtained according to the direct Fick method. The difference varied between 5 and 49 cc. per liter with a mean of 26.5 ± 2.79 and was not proportional to the value given by the Fick method. The third determination of arteriovenous oxygen difference in each subject was done after allowing a 20 minute interval between the two methods. If these are excluded, the dif-

TABLE I

Patient Age Sex	Diagnosis	O ₂ -consumption	B.M.R.	Arteriovenous O ₂ -difference		
				Catheter method	Grollman method	Difference Grollman- Catheter
		<i>cc./min.</i>	<i>per cent</i>	<i>cc./liter</i>	<i>cc./liter</i>	
1. 52 years M	Ess. hypertension	277 277	+27 +27	37 36	50 74	13 38
2. 24 years M	Normal	234 250	-10 -4	36 38 39	52 61 75	16 23 36
3. 23 years M	Normal	235 277	-10 +6	38 39 43	78 47 76	40 8 33
4. 24 years M	Normal	301 277	+15 -13	43 42 45	73 69 73	30 27 28
5. 26 years M	Ess. hypertension	291 331	+10 +25	26 27 28	54 60 44	28 33 16
6. 31 years F	Pregnancy mens V	207 179	+12 -3	32 33	43 44	11 11
7. 33 years F	Normal	255 233	+40 +28	35 21 24	40 66 71	5 45 47
8. 29 years F	Normal	243 240	+13 +12	31 40 37	80 71 53	49 31 16
Mean:				35.0	61.5	26.5 ± 2.79 N = 22

ference will be the same, or 26.5 ± 3.51 cc. per liter. This value is significant. The determination of arteriovenous oxygen difference according to the direct Fick principle thus gives values about .45 per cent lower than with the acetylene method.

In order to analyze the experimental errors involved, the following procedure was adopted:

The experimental error was defined as the variability of a single determination (δ_x) and calculated in two different ways:

The differences between determination 2 and 1, or 3 and 2, were computed for each method separately.

The differences 2 to 1 and 3 to 2 were of the same order of magnitude; thus all differences constituted a single group of data.

As the mean differences were not significant, the variability of a single determination may be calculated ac-

cording to the following formula:

$$\delta_x = \frac{1}{\sqrt{2}} \cdot \delta_d \quad (15)$$

where

δ_x = variability of single determination
 δ_d = standard deviation of difference

The results are given in Table II. The greatest differences between the determinations of the catheter method were -14 and +9, corresponding to an experimental error of 9.9 per cent (Table II). The value of -14 was found in patient No. 7, with basal metabolic rate of +40 per cent. This value falls outside the normal range of variation. If it is excluded the experimental error (Table II) will decrease to 5.6 per cent.

The experimental error was then calculated by a combination of analysis of variance (16), using Snedecor F-ratio as test of significance (17), dif-

TABLE II

*Experimental error of the catheter and Grollman method
(calculated per differences)*

(a) Catheter method

No. of diff.	Mean	Mean differences $D \pm \epsilon D$	Stand. deviation of diff. (δ_d)	Exp. error (δ_x)	
1	2	3	4	5	6
15	35.0	0.67 ± 1.26	ml. 4.88	ml. 3.46	per cent of mean 9.9
14	35.6	1.70 ± 0.75	2.81	1.99	5.6

(b) Grollman method

1	2	3	4	5	6
15	61.5	3.53 ± 4.39	17.0	12.1	21.3

ferentiating between the variation between individuals (δ_i) and within individual (δ_x) the latter corresponding to the experimental error, and "classical analysis."

The results are given in Table III.

TABLE III

*Experimental error of the catheter and the Grollman method
(calculated by analysis variance)*

	No. of indiv.	No. of determ.	Variability			
			Between individuals (δ_i)		Within individuals exp. error (δ_x)	
			ml.	per cent of mean	ml.	per cent of mean
Catheter	8	23	10.3	29.7	3.53	10.2
Grollman	8	23	15.3	24.4	13.3	21.2

The agreement between the two methods of calculation was excellent.

The catheter method was thus found to have a coefficient of variation for a single determination of 10 per cent (5.6 per cent), whereas the acetylene method, applied on the same patients under the same conditions during the simultaneous catheterization had a coefficient of variation of 21 per cent, which is significantly higher. The variability between the patients was found to be about the same for both methods (30, or 24 per cent).

There was no difference in oxygen content of the blood in the pulmonary artery sampled before, or 17 to 27 seconds after, the start of rebreathing. In eight cases the maximal difference between

the samples was 0.2 volumes per cent, the oxygen content being higher in some cases and lower in others during rebreathing.

The test for recirculation of acetylene showed that between five and 10 seconds after the start of acetylene breathing there was no acetylene in the pulmonary artery blood. However, in 10 to 15 seconds acetylene was present in the pulmonary artery blood, in amounts corresponding to an acetylene content in the alveolar air of 0.4 to 0.5 volumes per cent. In 15 to 20 seconds, this amount corresponded to approximately 1.5 volumes per cent.

DISCUSSION

In almost simultaneous determinations there was a statistically significant difference in arteriovenous difference and cardiac output of 45 per cent between values obtained with the direct Fick and the acetylene methods. This confirms earlier work in which separate determinations were done using these two methods. Bauman and Grollman have previously reported values with the acetylene method and the direct Fick that agreed fairly well (18). The mixed venous blood in their cases was sampled after puncture of the heart and the sampling done immediately after the rebreathing of acetylene, both factors making the results uncertain. The belief that the discrepancy between values for cardiac output obtained with heart catheterization and with the acetylene technique is due to apprehension produced by the catheterization is disproved by the result of this study. Instead, the difference must be due to some systematic error in the acetylene method.

Gladstone believed that the rebreathing technique augmented the circulation and thus gave rise to errors (9). The present study conclusively shows that there is no difference in oxygen content in the mixed venous blood during the short time of rebreathing as compared to that before rebreathing.

Hamilton, Spradlin and Saam, working with dogs, have shown (19) that the recirculation of acetylene is too rapid to permit waiting 25 seconds before the second air sample is taken. Rapid recirculation was confirmed by Starr and Collins, who used the dye injection technique in dogs (20).

Grollman and Bauman, on the contrary, after heart puncture in man, found that significant amounts of acetylene did not appear in the blood in the right ventricle until 25 seconds after the start of acetylene breathing (18). Hohwü Christensen was able to demonstrate that the acetylene content in the peripheral venous blood was very low and rose slowly, due to the diffusion of acetylene from the blood to the tissues (21, 22).

The present study shows that significant amounts of acetylene appear in the pulmonary artery blood between 10 and 15 seconds after the start of acetylene breathing. Since it is impossible to get full mixing and equalization of the acetylene within this time, it may be assumed that these factors are inherent sources of error in all methods using inhalation of foreign gas. This error becomes greater the longer the time elapsing before the second sample is taken. That recirculation is the main source of error is also shown by the fact that, in patients with slow circulation, *e.g.*, cardiac failure, gas inhalation methods give values for arteriovenous oxygen difference that equal those obtained with the direct Fick principle, 90 to 100 cc. per liter of blood (3, 13, 23). The results of dye injection experiments in man with the dye injected in the pulmonary artery and the right auricle also show that there is a significant recirculation within 10 seconds (24).

SUMMARY AND CONCLUSIONS

1. Almost simultaneous determinations of cardiac output according to the direct Fick (after heart catheterization) and the acetylene methods were made in eight subjects. A total of 22 sets of determinations were made.

2. There was a statistically significant discrepancy in the values obtained by the two methods for the AV oxygen difference. The mean difference averaged 26.5 ± 2.79 cc. per liter; the Fick method giving values about 45 per cent lower than the Grollman.

3. The experimental error for the Fick method was 5.6 per cent and for the acetylene method, 21 per cent.

4. The rebreathing procedure did not change the oxygen content in the pulmonary artery blood during the 27 seconds that the rebreathing lasted.

5. Tests for recirculation showed that within 10 to 15 seconds after the start of acetylene breathing

significant amounts of acetylene were found in the pulmonary artery blood.

6. Any method using inhalation of foreign gas will give values for arteriovenous oxygen difference which are too large, if the gas sampling is not completed before 10 to 15 seconds after the start of rebreathing. This error is not constant but, due to the speed of the circulation, is small or absent with slow circulation and is large with rapid circulation.

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A FACTOR DETERMINING THE LOCATION OF PSEUDOFRACTURES IN OSTEOMALACIA

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Narrow bands of diminished density, which resemble fracture lines and often appear symmetrically and last for months to years, have been described in the skeletons of individuals with a variety of diseases. The purpose of this paper is to offer a rational explanation for the bizarre locations of these pseudofractures as they occur in osteomalacia and to focus attention upon a possible mechanism for their occurrence.

The numerous descriptions of this phenomenon and the speculations as to its causes have been reviewed by Camp and McCullough (1). It has been described as a radiological syndrome by Milkman (2, 3). Histologically, such lesions in cases of rickets and osteomalacia were found by Looser (4, 5) to consist of infractions of the bones with subsequent laying down of non-calcified callus. Since they are not true fractures with complete separation of bone continuity, he called them *umbauzonen*, zones of alteration. He concluded that mechanical tension on softened bone, such as that due to muscle strain, perhaps caused small breaks in the cortex with gradual infractions through the rest of the bone, thus producing the long-lasting, symmetrical lines of diminished density resembling fracture lines. Callus about true fractures remains uncalcified for a long time in cases of osteomalacia, and Looser felt that it was not strange for these *umbauzonen* to persist for a considerable time after their first appearance.

Albright and coworkers (6) have reviewed the cases in which pseudofractures have been found. They state that the narrow decalcified bands which occur in otherwise normal-appearing bone, and which tend to be symmetrical and remain stationary for months to years, occur only in osteomalacia or rickets. They point out that the underlying bone disturbance in the variety of conditions where

these bands of diminished density have been noted, *i.e.*, rickets, late rickets, celiac disease, chronic idiopathic steatorrhea, sprue, and severe acidosis, is really osteomalacia. The lesions are common in advanced cases of hunger osteopathy. Somewhat similar bands occur in Paget's disease, congenital syphilis, marble bones, osteogenesis imperfecta and hyperparathyroidism, but in these conditions the lesions are rarely symmetrical and occur through areas of definite bone abnormality as seen by X-ray. "March fractures" appear in otherwise normal bones but are rarely symmetrical and readily unite and become calcified. As for the mechanism of production of pseudofractures,



FIG. 1. SCAPULA SHOWING PSEUDOFRACTURE

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Albright and coworkers refer to the work of Looser (4).

During the past year on the Radiological service of the Presbyterian Hospital, we have seen four patients with these narrow bands of lessened density. They all showed blood findings consistent with osteomalacia, *i.e.*, normal calcium, low phosphorus and elevated alkaline phosphatase. One was a case of long-standing osteomalacia of undetermined origin; the other three showed the syndrome, described by Albright and coworkers (6), of renal acidosis without nitrogen retention. Pseudofractures occurred in similar locations in the bones of these four individuals. As in the published cases, the most common sites for these lesions were in the axillary border of the scapula (Figure 1), the ribs, the pubic rami, and the upper end of the femur. They also occur in the iliac bones (Figure 2), and near the base of the spine of the scapula (Figure 3).

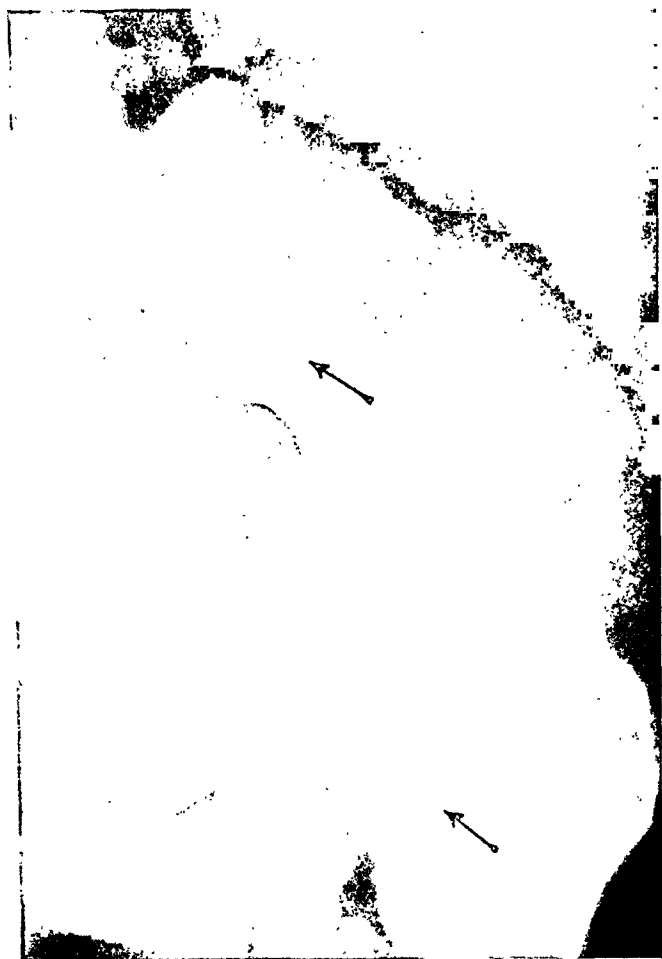


FIG. 2. ILIUM AND THE NECK OF A FEMUR SHOWING PSEUDOFRACTURES

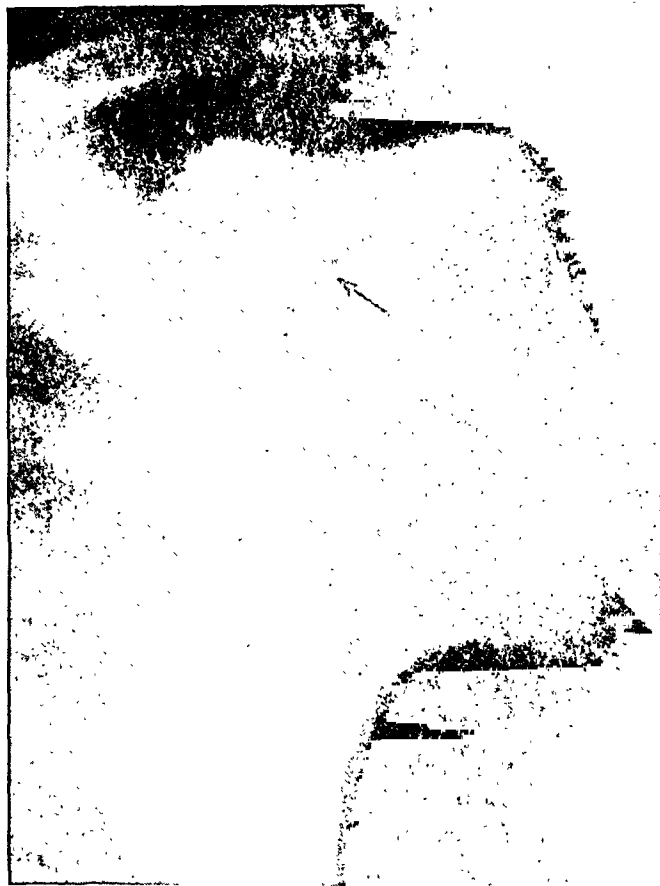


FIG. 3. PSEUDOFRACTURE OCCURRING IN THE SPINE OF A SCAPULA. A SYMMETRICAL LESION IS PRESENT IN THE OPPOSITE SCAPULA

The pseudofractures in the scapulae and the iliac bones, particularly, occur in areas where muscle strain seems unlikely. Since these lesions tend to develop regularly in the same places in different patients and tend to be symmetrical on the two sides of the body, an anatomical explanation for the lesions was sought.

CLINICAL MATERIAL AND METHOD

Complete skeletal radiographs were made of the four patients mentioned above. The reproductions of radiographs in cases reported in previous studies (2, 3, 6-11) were reviewed and in 28 of these cases the illustrations were adequate to show accurate localization of the lesions. Dissection was made of the blood vessels supplying the trunks and extremities of three cadavers to ascertain the proximity of the main vessels to the bones.

RESULTS

The sites where pseudofractures have been found, and the arteries which commonly cross the bones at these sites, are shown in the two halves of Figure 4. The number of times lesions were found

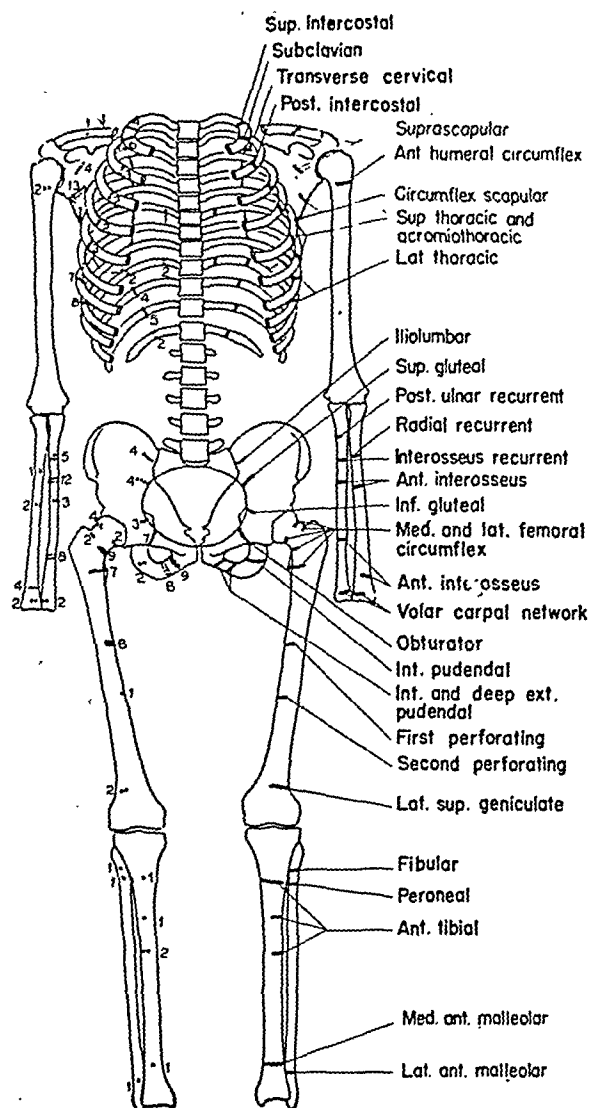


FIG. 4. SKELETON SHOWING THE USUAL SITES OF OCCURRENCE OF PSEUDOFRACTURES AND THE ARTERIES WHICH COURSE THE BONES AT THESE SITES

The figures signify the number of lesions found at these locations in four cases at the Presbyterian Hospital and the lesions pictured in previous studies (2, 3, 6-11).

at these sites in our four cases and in which they were adequately pictured in the cases reported previously is indicated on the figure. The arteries shown are those coursing directly over the bones at the sites of the lesions as given in standard anatomy works (12, 13). All of these arteries were found by dissection to lie in contact with the bone. The blood vessels in the hands and feet vary considerably in different individuals in relation to the bones and therefore were not included in the figure.

DISCUSSION

Pseudofractures are not infrequent in the first rib where the bone is normally notched by the subclavian artery. In the axillary border of the scapula where the circumflex scapular artery grooves the bone, 13 pseudofractures were found. A detailed view of the blood vessels around the scapula is shown in Figure 5, and the close correspondence between the locations of these blood vessels and the scapular lesions shown in Figure 4 will be noted. A pseudofracture has also been described (3) in the outer end of the acromion at the site of the vessel shown in Figure 5.

Careful positioning of the patient is required at times to demonstrate some of the pseudofractures, particularly those in the ribs and scapulae. To be seen clearly, small incomplete fractures in normal bone due to trauma also require careful positioning of the patient. Both types of lesions are more distinct when they occur perpendicular to the long axis of the trabecular pattern of the bone. This could explain the failure of lesions to be common where the radial artery spirals the humerus. We have seen only one case (14), that of a severe rachitic infant, with bilateral symmetrical spiral lesions in the midportion of the humerus. Gross examination of normal scapulae shows that some of them are not grooved by the circumflex scapular artery. Variation in the course of this blood vessel may explain why pseudofractures are not

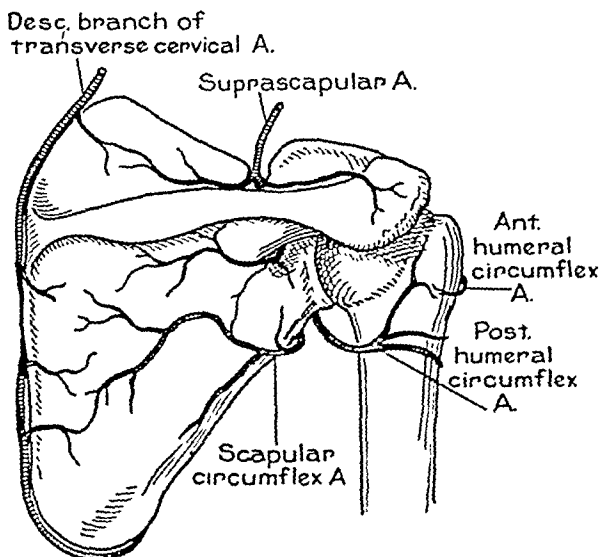


FIG. 5. SCAPULA SHOWING THE POSITION OF THE BLOOD VESSELS IN RELATION TO THE BONE

always seen at this site. Similar variations of other blood vessels may produce the individual differences observed. The lesions in ribs nine, 10, 11, and 12, and the posterior lesion in rib six could be at the site of branches of intercostal arteries. Vessels were found in these areas in one of three cadavers examined.

Mechanical stress, such as that due to muscular or postural strain, may produce true fractures at the site of pseudofractures, particularly in the upper end of the femurs in the far advanced cases. It seems doubtful that this type of strain would account for most of the lesions initially. Fractures from other mechanical causes do not often occur at the sites commonly affected in osteomalacia, as demonstrated in Cushing's disease by the fractures in the anterior ends of the lower ribs where respiratory strain on the thoracic cage is greatest. The true fractures, and even some of the pseudofractures, occurring in cases of osteomalacia may be due to other kinds of mechanical stress, but most of the lesions are at sites where muscular or postural stress is unlikely, and where arteries lie in close contact with the bones. The most likely explanation for the occurrence of these lesions here would be some type of vascular stress.

Normal bone tends to yield to continuous pressure in a very definite way as is clearly demonstrated in the skull by patterned thinning due to the pressure of convolutions of the brain and in other bones by grooving due to pressure of blood vessels. Although the exact mechanism of bone erosion at sites of pressure is not known, it is not surprising that malacic bones should respond differently to stress than normal bones. Small breaks in the cortex of bones at the site of mechanical stress due to pulsating blood vessels, rather than muscular strain as suggested by Looser, would be followed by the laying down of callus. As osteomalacia is characterized by failure of deposition of calcium in newly formed osteoid tissue, the callus would remain uncalcified in these sites and account for the radiological and pathological appearance of the pseudofractures. That there is selective resorption, or lack of deposition of calcium adjacent to the large blood vessels, seems less likely. It is to be hoped that the correlation between the locations of blood vessels and sites of pseudofractures given here will initiate attempts at pathological or operative verification, and experi-

mental study of the mechanism of this phenomenon in osteomalacia and possibly other bone diseases.

The pressure of vessels upon the bones may also account for some of the similar bands of decreased density occasionally appearing in the skeletons of patients with other conditions, such as osteogenesis imperfecta, marble bones, and Paget's disease, but these lesions occur in abnormal bone, as seen by X-ray, and their mechanism and character would vary with the disease and especially with the ability to calcify the callus formed.

SUMMARY

The location of the narrow bands of diminished density which often appear symmetrically and last for months to years in the skeletons of patients with osteomalacia, is shown to correspond with the location of main blood vessels which lie on the bones. It is suggested that the mechanical stress caused by the vessels may result in small breaks in the cortex with subsequent laying down of callus which, in osteomalacia, is uncalcified. This mechanism explains the symmetrical appearance of the pseudofractures in otherwise normal appearing bone and their location at sites not usually subjected to other mechanical stresses.

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THE OXYGEN CONSUMPTION OF THE NORMAL AND THE DISEASED HUMAN KIDNEY¹

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The development of a technique of intravenous catheterization and its application to the sampling of renal venous blood (1) have made possible the study of the metabolic activity of the kidney in the unanesthetized human subject. We have used this method of investigation in conjunction with the usual clearance procedures in an attempt to determine the oxygen consumption of the normal kidney and its variation in disease.

METHODS

All subjects were studied at rest and in the post-absorptive state. Inulin and sodium para-aminohippurate (PAH) clearances were performed as described by Goldring and Chasis (2), urine being collected by bladder catheterization. Following the injection of the priming doses and the starting of the sustaining infusion of inulin and PAH, a modified ureteral catheter was introduced into an antecubital vein of the opposite arm, and passed under fluoroscopic visualization through the right atrium and inferior vena cava and into the right renal vein. The rotation of the curved tip of the catheter posteriorly, and other details of technique have been described by Bradley (3), and by Warren, Brannon, and Merrill (1). Urine was collected at accurately timed intervals of 10-30 minutes, and at approximately the midpoint of each urine collection period blood samples were obtained from a femoral artery and the right renal vein. There was usually an interval of one or two minutes between the withdrawal of these samples. A portion of each sample was collected anaerobically, either under mineral oil, or in an oiled heparinized or citrated syringe, and the oxygen content determined by the method of Van Slyke and Neill (4). The remainder of the samples were oxalated and centrifuged as soon as possible for the determination of the plasma inulin and PAH content by the methods of Corcoran and Page (5) and Smith *et al.* (6). From two to six urine collection periods and a corresponding number of pairs of arterial and renal venous blood samples were obtained from each subject. In addition, paired samples of heparinized and oxalated whole blood were obtained from each subject for hematocrit and oxygen capacity determinations.

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Clearances of inulin and PAH were calculated in the usual manner from the rate of urinary excretion and the arterial levels of these substances. The percentage extraction of PAH was determined by the formula $\frac{A-R}{A} \times 100$, where A and R represent the PAH concentrations in arterial and renal venous plasma, respectively, and the renal plasma flow obtained by dividing the clearance of PAH by the extraction: $RPF = \frac{C_{PAH}}{\text{Ext. PAH}}$. A discussion of the calculation of renal plasma flow in this manner is presented in a separate publication (7). This figure was corrected for the hematocrit reading to obtain the renal blood flow. The difference in oxygen content between arterial and renal venous blood (renal oxygen extraction), expressed as volume of O₂ per cc., was multiplied by the renal blood flow to obtain the renal oxygen consumption: Renal oxygen consumption = RBF × A_{O₂} - R_{O₂}.

Van Slyke *et al.* (8) have maintained that the arterial-renal venous oxygen difference is not always a true measure of renal oxygen extraction in the dog, since they found definite differences in the hemoglobin content (oxygen capacity) of arterial and renal venous blood in that animal. In 22 comparisons of the oxygen capacities of paired arterial and renal venous samples we have found a mean difference of 0.2 volume per cent, with a standard error of $\pm .01$. Seventy-one comparisons of paired arterial and renal venous hematocrit determinations showed a mean difference of 0.1 per cent packed red cell volume, with a standard error of .07. These differences are well within the limits of technical error.

Since the arterial-renal venous oxygen difference is quite small, and must be multiplied by a large figure for renal blood flow to obtain the oxygen consumption, the measurement of this difference constitutes a potential source of grave error. All oxygen determinations were done in duplicate and discarded if they did not agree within 0.1 volume per cent. Successive samples of arterial and renal venous blood in a given patient over a one or two hour period usually showed minor fluctuations in oxygen content, the mean difference between successive determinations of A-V differences in 31 subjects being 0.05 volume per cent. It is felt that these variations are partly real and partly due to technical error; in any case, they are not of sufficient magnitude to compromise the conclusions we have drawn from our observations.

All figures have been corrected to a standard surface area of 1.73 sq. m.

RESULTS

Thirty-six subjects have been studied in this manner. (Additional studies on most of these subjects have been presented in a separate publication [7]). For the presentation of the data they have been divided into four groups, according to the clinical classification of their disease. The first group (Table I) consists of ten patients in whom no organic disease could be demonstrated or who were suffering from a chronic illness unrelated to the kidney. The clinical impression of normal renal function in these subjects was confirmed by the demonstration of normal values for glomerular filtration rate and renal blood flow as established by others. The renal oxygen extraction (arterial-renal venous oxygen difference) is extremely small in comparison with arterio-venous differences elsewhere in the body, ranging from 1.09 to 1.87 volumes per cent, with a mean value of 1.42 volumes per cent. Although the amount of oxygen removed from each unit of blood perfusing the kidney is quite small, the renal blood flow is so large that the total renal oxygen consumption is of considerable magnitude, ranging from 12.0 to 20.8 cc./min., with a mean value of 16.0 cc./min.

The second group of subjects consists of patients whose predominant complaint was hypertension, and in whom a history of previous renal disease could not be elicited. In some of them renal damage was clinically demonstrable, as evidenced by albuminuria, reduced phenolsulfonathalein excretion, or hyposthenuria. On purely clinical grounds it was presumed that the impaired renal

TABLE I
Normal subjects

Pt.	CIN	FF	RBF	O ₂ Ext.	O ₂ Cons.
	cc./min.		cc./min.	vols. %	cc./min.
W. F. W.	120	.14	1456	1.10	16.0
A. J.	115	.17	1057	1.13	12.0
W. W.	106	.14	1284	1.09	14.0
E. W.	107	.16	932	1.73	16.1
M. C.	142	.17	1450	1.32	19.2
R. M.	133	.18	1155	1.44	16.6
R. B.	92	.15	1029	1.87	18.2
J. H.	131	.23	948	1.53	14.8
R. A.	115	.18	1401	1.48	20.8
J. R. H.	118	.23	835	1.46	12.2
Mean	117.1	.18	1155	1.42	16.0
S.D.	±14.7	±.04	±229	±.25	±2.9

TABLE II
Essential hypertension and nephrosclerosis

Pt.	CIN	FF	RBF	O ₂ Ext.	O ₂ Cons.
	cc./min.		cc./min.	vols. %	cc./min.
I. S.	126	.17	1196	0.82	9.8
O. W.	140	.19	1237	1.81	22.4
C. M.	143	.22	1160	1.49	17.3
B. G.	133	.24	971	1.18	11.5
C. E.	70	.23	535	1.96	10.5
E. T.	97	.28	590	1.54	9.1
M. S.	109	.25	701	1.51	10.6
B. H.	36	.19	247	1.91	4.7
G. B.	10	.07	212	2.09	4.4
I. P.	97	.18	852	1.37	11.7
R. L.	109	.23	784	0.98	7.7
Mean	97	.21	771	1.51	10.9
S.D.	±32	±.02	±357	±.41	±4.2

function in these subjects was secondary to the hypertension, and that the diagnosis of nephrosclerosis was applicable. It will be seen from Table II that the renal blood flow varied in this group from normal to extremely low levels. The oxygen extractions did not vary correspondingly, the mean, 1.51 volumes per cent, being not significantly different from the normal mean.

The six subjects listed under Table III as chronic nephritis were patients who gave histories of long-standing kidney disease and who were found to have severe impairment of renal function. Of these, N. W. and L. H. probably represented chronic pyelonephritis, the others chronic glomerulonephritis, although the clinical differentiation of these diseases was open to question. In spite of the marked reduction in renal blood flow in this group, to a mean value of 353 cc./min., the renal oxygen extractions were within the normal range.

TABLE III
Chronic nephritis

Pt.	CIN	FF	RBF	O ₂ Ext.	O ₂ Cons.
	cc./min.		cc./min.	vols. %	cc./min.
J. M.	56	.12	636	1.84	11.7
E. M.	19	.11	245	1.30	3.2
B. R.	17	.07	347	1.74	6.0
N. W.	50	.18	482	1.72	8.3
L. H.	20	.18	168	1.60	2.7
M. L.	21	.12	242	1.90	4.6
Mean	30.5	.13	353	1.68	6.1
S.D.	±17.0	±.04	±176	±0.20	±3.3

TABLE IV
Clinical data on patients with glomerulonephritis

Pt.	Age	Duration	Edema	B.P.	Fundi	Plasma			Hgb	Urine			Remarks
						Albumen	Cholesterol	NPN		Protein	RBC	Casts	
T. B.	22	6 weeks	1+	135/85	Normal	3.6	260	31	13.4	3+	2+	2+ granular	Weakness; severe lumbar pain
G. W.	37	8 weeks	4+	116/70	Normal	1.5	640	33	15.0	4+	0-1+	2+ hyaline & waxy	Asymptomatic except for massive edema
D. R.	49	8 weeks	3+	160/100	Normal	1.7	300	52	13.0	3+	0-1+	1+ granular & hyaline	Nausea and vomiting. One convulsion
A. H.	38	5 weeks	1+	145/85	Arteriolar narrowing	1.6	440	30	13.0	3+	2+	2+ granular & RBC	Headache, blurred vision
B. B.	39	6 mos.	0	124/88	Normal	2.4	200	35	10.0	1+	1+	1+ granular	Arthritis
L. D.	31	8 weeks	2+	130/95	Normal	3.0	220	40	12.0	2+	2+	2+ granular & RBC	Headache
G. I.	38	5 mos.	3+	145/95	Normal	1.7	720	36	15.0	2+	0-1+	1+ granular	Weakness, headache
R. E.	42	6 mos.	0	200/140	Arteriolar narrowing	2.9	250	38	18.0	1+	1+	1+ granular & RBC	Headache, occ. blurred vision
W. A.	21	2 years	1+	150/70	Normal	3.8	180	49	16.0	1+	1+	1+ granular	Rheumatic heart disease

Significant clinical data on ten patients who were diagnosed as acute or subacute glomerulonephritis are summarized in Table IV. Two of these patients (G. W. and G. I.) were obviously in the nephrotic or "degenerative" stage of the disease, as demonstrated by massive edema, proteinuria and extreme hypoalbuminemia and hypercholesterolemia. In the others, hypertension, hematuria, or a combination of all elements of the disease predominated. W. A. had an attack of hemorrhagic nephritis of several weeks' duration two years preceding the time of study. He had been asymptomatic until one week before admission when he suffered a mild upper respiratory infection, and puffiness of the eyelids and microscopic hematuria were noted. It is felt that he represents an acute exacerbation of a latent glomerulonephritis. The clinical course of patient R. E. was more suggestive of essential hypertension than glomerulonephritis; he was placed in

this group because of albuminuria, hematuria, and the discovery of red blood cell casts in his urinary sediment. It is our impression that these findings do not accompany primary hypertension except in the malignant phase.

A consistent finding in all of these patients was a greater reduction in glomerular filtration rate than in renal blood flow, so that the filtration fraction was uniformly low. This is suggestive of primary glomerular disease, and is in keeping with the findings of Earle, Taggart, and Shannon (8). Likewise, the normal values for renal blood flow indicate that the disease process was not of long standing.

It may be seen from Table V that the renal oxygen consumption of these patients varied between extreme limits, from 0.9 cc./min. to 13.8 cc./min. Since the blood flows were normal or only slightly reduced, this variation was due almost entirely to changes in oxygen extraction. This is in con-

trast to the preceding groups, in which variations in blood flow and normal oxygen extractions were found. No correlation existed between the predominant symptomatology in any patient and the oxygen consumption of his kidneys. It is true

that patient D. R., whose renal oxygen consumption was extremely low, was also the most severely ill; patient B. B., however, whose kidneys were also consuming comparatively little oxygen, was ambulatory at the time of study. Although the

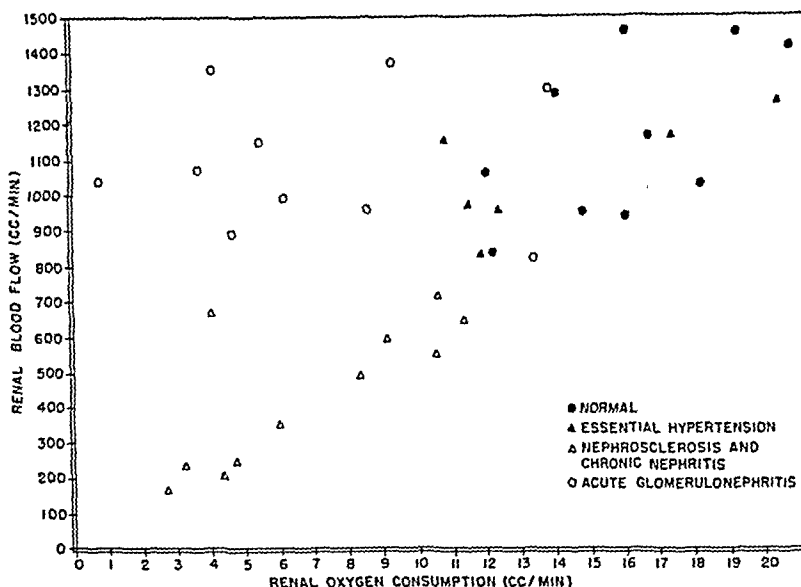


FIG. 1. RENAL BLOOD FLOW AND RENAL OXYGEN CONSUMPTION IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPERTENSION, NEPHROSCLEROSIS AND ACUTE GLOMERULONEPHRITIS

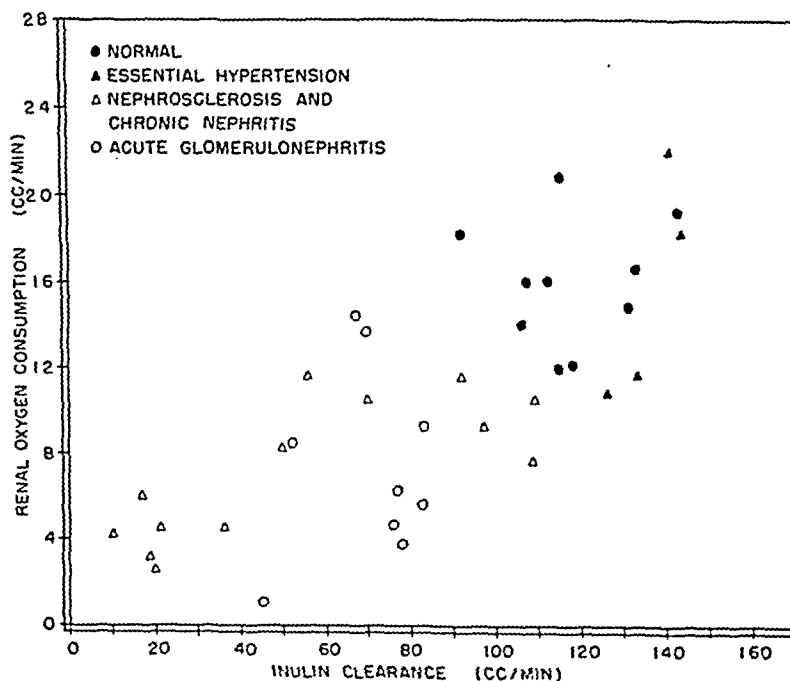


FIG. 2. RENAL OXYGEN CONSUMPTION AND GLOMERULAR FILTRATION RATE IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPERTENSION, NEPHROSCLEROSIS AND ACUTE GLOMERULONEPHRITIS

TABLE V
Acute and subacute glomerulonephritis

Pt.	C _{IN}	FF	RBF	O ₂ Ext.	O ₂ Cons.
	cc./min.		cc./min.	vols. %	cc./min.
T. B.	83	.12	1141	0.48	5.5
G. W.	67	.13	816	1.64	13.4
D. R.	45	.08	1041	0.09	0.9
A. H.	77	.12	994	0.62	6.2
B. B.	78	.10	1065	0.35	3.7
L. D.	71	.11	1293	1.07	13.8
G. I.	83	.10	1362	0.68	9.3
W. A.	51	.09	959	0.90	8.6
R. E.	76	.16	887	0.53	4.7
Mean	70	.11	1062	0.71	7.3
S.D.	±14	±.03	±179	±0.45	±2.5

oxygen data in two of these subjects were normal, we consider it noteworthy that the mean values for both oxygen extraction and renal oxygen consumption for the entire group are distinctly below the normal range.

The data may be represented graphically as in Figure 1, where renal blood flow is plotted against oxygen consumption. The scatter in the normal range is large, but it is clearly evident that as the renal blood flow falls in chronic renal disease there is a corresponding fall in renal oxygen consumption. In contrast, the majority of the patients with acute and subacute glomerulonephritis are distinguished by a low oxygen consumption with a normal renal blood flow. This difference disappears when oxygen consumption is plotted against glomerular filtration rate, as in Figure 2. Although there is no apparent relation between these factors in any one group, a definite correlation ($r = .76$) exists when all 36 subjects are considered.

DISCUSSION

The rate of oxygen utilization by an organ is generally considered to be an expression of its metabolic activity. The metabolism of the normal mammalian kidney has been the subject of numerous investigations (9-12), all of which have demonstrated that renal oxygen consumption is quite high in relation to the weight of the organ, and that this high oxygen consumption is the result of a small arterio-venous oxygen difference (oxygen extraction), and a large renal blood flow.

Van Slyke *et al.* (9) and others (11, 12) have observed in the dog that renal oxygen extraction

remains constant when renal blood flow is varied, so that the rate of oxygen consumption is a function of blood flow. Similar results have been obtained in man by Bradley and Halperin (13), who used the technique of intravenous catheterization to determine renal oxygen consumption before and during reduction of renal blood flow by abdominal compression. These authors also observed that the reduced oxygen consumption found during the period of decreased blood flow was associated with apparent cessation of urine formation in a certain proportion of nephrons, and suggested the possibility that "renal oxygen consumption depends upon the relative proportion of tubular tissue actively functioning in the formation of urine from glomerular filtrate."

In this view, it seems logical to expect that renal oxygen consumption would vary with filtration rate if the amount of functioning tubular tissue remained unchanged. Our data show that the kidneys of patients with acute and subacute glomerulonephritis, which had low rates of glomerular filtration, also had low rates of oxygen utilization, in spite of normal or increased blood flow. Earle, Taggart, and Shannon (8) have shown that although the total amount of functioning tubular tissue (as estimated from diodrast Tm) tends to fall with the filtration rate, the ratio of filtration rate to Tm is lower than normal in the early stages of glomerulonephritis, so that there is a greater depression of filtration than of tubular function. It is conceivable that the observed reduction in renal oxygen consumption in our patients was due to decreased formation of glomerular filtrate. An alternative explanation is that in acute and subacute glomerulonephritis there is a fundamental alteration in the metabolic activities of renal cells which results in a diminished utilization of oxygen.

It is of interest in this connection that we have found a definite correlation between filtration rate and oxygen consumption in all our subjects (Figure 2).

The progressive vascular occlusion and destruction of renal parenchyma which result in the granular, contracted kidneys of nephrosclerosis and chronic nephritis are accompanied by decreases in both blood flow and oxygen consumption (Figure 1). It is probable that the diminished oxygen consumption found in these patients is simply a reflection of the lowered metabolic demands of the

functionally smaller kidneys, the volume of blood flow being an expression of the amount of surviving tissue.

Merrill (14) has found that the decreased renal blood flow accompanying congestive heart failure is compensated for by an increased oxygen extraction, so that oxygen consumption remains normal. (We have found extractions of 3.6 and 4.2 volumes per cent, values significantly greater than our normal mean, in two subjects with congestive failure who are not included in the present series.) Glomerular filtration rate was also reduced in these patients. If filtration rate determines tubular work, and therefore renal oxygen consumption, it is necessary to suppose that independent tubular activity is increased in congestive heart failure.

Although the kidneys of the patients with acute and subacute glomerulonephritis were consuming comparatively little oxygen, they were carrying on all of the functions which are generally considered to constitute renal work, including the reabsorption of water and electrolytes against osmotic gradients, the excretion of urea, and the tubular secretion of PAH. The disproportion between the oxygen consumption of the kidney and the theoretical thermodynamic requirements of known renal work has been pointed out by Barcroft and Brodie (10), Glaser *et al.* (11), and Van Slyke *et al.* (9). These last investigators concluded that "the overwhelmingly greater part of the energy produced must be utilized by the kidney for its own internal cellular processes not related to the external work which the organ is performing." Since the nature of these internal cellular processes is completely obscure, it is not inconceivable that their activity, and consequently the demand of the kidney for oxygen, may vary within wide limits without being detectable by known methods of measurement.

In view of the extensive speculation concerning the relation of renal ischemia to essential hypertension our observations in patients with this disease are of interest. Pathologists agree that longstanding hypertensive disease is usually associated with progressive narrowing and even occlusion of small renal blood vessels, particularly the preglomerular arterioles. The functional counterpart of this constriction of the renal vascular bed is evident in some of our patients, in whom definite reductions of renal blood flow were found.

Since the decreased blood flow was not accompanied by an increased oxygen extraction, the oxygen consumption of these kidneys was also low.

The question of whether these vascular changes precede or follow the hypertension has been the subject of much debate. In four patients with sustained hypertension of at least six months' duration we have found normal values for renal blood flow and oxygen consumption, which demonstrates that in at least some cases of essential hypertension the metabolic activities of the kidney are carried on at the normal level.

SUMMARY AND CONCLUSIONS

1. Renal oxygen consumption in unanesthetized human subjects has been estimated by combining the techniques of intravenous catheterization and sodium para-aminohippurate clearance.
2. The mean normal renal oxygen extraction was found to be 1.42 volumes per cent, and the mean normal renal oxygen consumption 16.0 cc./min.
3. Decreased levels of oxygen consumption were found in patients with nephrosclerosis, chronic glomerulonephritis, and chronic pyelonephritis, who also had diminished renal blood flow. Oxygen extraction was normal in these patients.
4. In patients with acute and subacute glomerulonephritis the extraction of oxygen was decreased in spite of a normal or increased renal blood flow.

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THE MEASUREMENT OF GLOMERULAR AND TUBULAR PLASMA FLOW IN THE NORMAL AND DISEASED HUMAN KIDNEY¹

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The clearance of para-aminohippuric acid (PAH) has been extensively used as a measure of the rate of renal plasma flow. The validity of this measurement depends upon the assumption that at low plasma levels PAH is completely extracted from the blood by tubular cells, and that therefore the only limiting factor to its rate of excretion in the urine is the rate at which it is delivered to the tubules. There is ample proof that this assumption is justified in normal man under basal conditions (1, 2). However, in any condition in which the total number or the functional capacity of tubular cells is decreased, the complete extraction of PAH and therefore the use of the clearance alone as a measure of renal plasma flow is open to serious question. The uncertainty as to the degree of renal impairment which is compatible with complete extraction of PAH has hampered the application of this method to the study of the diseased human kidney.

According to the Fick principle, the blood flow through an organ may be determined if the rate of consumption (or excretion) of a substance and the concentration of this substance in blood entering and leaving the organ (arterial-venous difference) are known. The application of the technique of venous catheterization to the sampling of blood from the renal vein in man (1) has made possible the utilization of this principle to determine the actual renal blood flow under conditions in which the extraction of PAH may not be completed.

In the normal kidney histological studies indicate that under most circumstances the blood flow through the tubules equals the blood flow through the glomeruli. In the diseased kidney either the glomeruli or tubules may be destroyed separately and there is no assurance from

histologic studies that glomerular flow equals tubular flow. The glomerular flow can be measured from the rate of inulin excretion and the arterio-renal venous difference of inulin and compared with the tubular flow measured at the same time by the PAH excretion and arterio-renal venous PAH difference.

METHODS

The standard clearance procedures described by Goldring and Chasis (3) have been followed. Priming and sustaining infusions of inulin and sodium para-aminohippurate were given and urine collected by bladder catheterization at accurately timed intervals of 10-30 minutes. The dosage of PAH was adjusted to achieve an arterial blood level between 1 and 3 mg. per cent. An intravenous catheter was introduced into the right renal vein according to the procedure described by Warren, Brannon and Merrill (1). At approximately the midpoint of each urine collection period samples of renal venous and femoral arterial blood were withdrawn. The inulin and PAH content of plasma and urine were determined by the methods of Corcoran and Page (4) and Smith *et al.* (5), corrections being made for inulin blanks in plasma and urine. A 1:5 cadmium sulfate filtrate was used for PAH determinations in renal venous plasma instead of the 1:15 filtrate for arterial plasma. Paired samples of heparinized blood were obtained from each patient for hematocrit determinations.

CALCULATIONS

The application of the Fick principle to the determination of renal plasma flow in the dog from the excretion and extraction of PAH has been discussed by Phillips *et al.* (6). As we have not found any significant difference between arterial and renal venous hematocrit readings, nor between the PAH concentration of whole blood and plasma, we have not applied the corrections these authors suggest, but have used simply the formula (1)

$$RPF = \frac{UV}{A - R}$$
where UV is the rate of urinary excretion of PAH (mg./min.), and A and R represent the PAH concentrations in arterial and renal venous plasma (mg./cc.). The extraction of PAH was determined as the quotient of the arterial-renal venous PAH difference and the arterial level: (2)
$$Ext. = \frac{A - R}{A}$$
and multiplied by 100 to express it as a percentage. The clearance

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of PAH was calculated in the usual manner from the rate of urinary excretion and the arterial level: (3)

$C_{PAH} = \frac{UV}{A}$. It is evident from the integration of equations (1), (2) and (3) that the renal plasma flow may be obtained by dividing the clearance of PAH by its extraction: (4) $RPF = \frac{UV}{A - R} = \frac{C_{PAH}}{\text{Ext. PAH}}$.

Since inulin is a foreign substance which is neither metabolized nor stored by the kidney, substitution of inulin concentrations in the above formulae should also yield a valid estimate of renal plasma flow. According to the available evidence, inulin is excreted entirely by glomerular filtration, whereas the excretion of PAH is accomplished largely by active tubular transfer. The value for renal plasma flow obtained from inulin concentrations should represent therefore only the volume of plasma which perfuses the glomeruli and, derived from PAH clearance and extraction, the flow through both glomeruli and tubules, predominantly the latter. These determinations may therefore be designated glomerular plasma flow (GPF) and tubular plasma flow (TPF).

The filtration fraction (FF) represents the fraction of plasma water which is filtered at the glomerulus, and has been determined by us as the ratio of the inulin clearance to the tubular plasma flow: $FF = \frac{C_{IN}}{TPF}$. If glomerular and tubular plasma flows are equal, the filtration fraction should equal the inulin extraction as obtained from Equation (2).

This derivation of the filtration fraction differs from that usually given, as other investigators have used this term to represent the ratio of the inulin clearance to the PAH clearance alone. Our values for FF are therefore lower than those previously reported.

All figures have been corrected to a surface area of 1.73 sq. m.

RESULTS

Data on ten subjects who were hospitalized for minor illnesses unrelated to the kidney, and

who had normal blood pressures, are summarized in Table I. The subjects with abnormal renal function have been divided into two groups: those in whom vascular changes might be considered primary, and those in whom disturbances of renal circulation might be considered secondary to glomerular dysfunction. The first group (Table II) includes patients whose predominant complaint was high blood pressure. In some of these impairment of renal function was manifested clinically by albuminuria, hyposthenuria, and decreased phenolsulfonphthalein excretion. Others presented only a history of elevated blood pressure for six months or more. Two patients who were diagnosed clinically as chronic pyelonephritis are also included in this group.

The second group (Table III) is composed entirely of subjects with acute, subacute or chronic glomerulonephritis.

There are many possible objections to this division of subjects. The variety of structural changes which may be produced in the kidney by any one of these disease processes is reflected in the disagreement of authorities as to their pathological classification. The existence of diffuse arteriolar disease in chronic glomerulonephritis is well recognized, as is the fact that extensive glomerular changes occur in nephrosclerosis. A functional basis for the clinical classification of these patients is revealed, however, in the values found for the filtration fraction. As demonstrated in Figure 1, there is a sharp division between the patients with hypertensive disease of the kidney and those with glomerulonephritis; the former lying above, and

TABLE I
Normal subjects

Pt.	C_{IN}	Inulin extraction	GPF	C_{PAH}	PAH extraction	TPF	FF	Diagnosis
	cc./min.	per cent	cc./min.	cc./min.	per cent	cc./min.	per cent	
E. F.	112	24.6	450	544	83	659	17.0	Bromide Intoxication. (Convalescent)
A. J.	115	11.6	990	595	89	668	17.0	Rheumatoid Arthritis
W. W.	106	14.5	730	716	93	770	14.0	Multiple Sclerosis
E. W.	107	14.1	759	595	91	653	16.4	Psychoneurosis
R. B.	92	19.7	467	519	86	604	15.2	Fibromyositis
J. H.	131	20.7	633	527	92	573	22.9	Peptic Ulcer
L. G.	112	12.3	895	730	92	794	13.9	Psychoneurosis
M. A. C.	142	—	—	764	91	840	16.9	Peptic Ulcer
R. M.	133	—	—	652	90	725	18.2	Primary Optic Atrophy
W. F. W.	120	—	—	742	90	825	13.6	Multiple Sclerosis
Mean	117	16.8	703	638	90	710	16.5	

TABLE II
Vascular diseases

Pt.	CIN	Inulin extraction	GPF	CPAH	PAH extraction	TPF	FF	Diagnosis
	cc./min.	per cent	cc./min.	cc./min.	per cent	cc./min.	per cent	
R. L.	109	21.5	507	421	90	468	23.3	Essential Hypertension
C. M.	143	26.3	544	588	90	654	21.9	Essential Hypertension
B. G.	133	17.2	773	494	91	545	24.4	Post-Toxic Hypertension
M. S.	109	21.4	509	376	87	432	25.2	Essential Hypertension
E. T.	97	26.3	369	276	80	345	28.1	Nephrosclerosis
C. E.	70	25.7	272	266	86	310	22.6	Nephrosclerosis
L. M.	54	14.1	383	222	76	292	18.5	Nephrosclerosis
N. W.	50	17.9	280	206	75	275	18.2	Chronic Pyelonephritis
B. H.	36	20.3	177	110	58	190	19.0	Nephrosclerosis
G. H.	36	15.0	240	102	64	159	22.6	Nephrosclerosis
L. H.	20	10.3	194	83	73	114	17.5	Chronic Pyelonephritis
Mean	78	19.6	386	285	79	344	21.9	

the latter below the normal mean. The two patients with chronic pyelonephritis were found to have filtration fractions similar to the patients with nephrosclerosis with comparable degrees of renal damage.

It may be seen from Tables II and III that the extraction of PAH remains within the normal range (83-93 per cent) until renal damage is moderately advanced. The number of determinations is insufficient to allow quantitative assessment of the degree of renal impairment which is incompatible with the complete extraction of PAH at low plasma levels. It appears, however, from these data that PAH extraction does not begin to decrease until the inulin clearance has fallen below

60 cc./min. and PAH clearance below 300 cc./min. Figure 2 is a graphic representation of the latter relationship.

Estimates of glomerular and tubular plasma flow, as determined from the clearance and extraction of inulin and PAH, are given in the tables and compared in Figure 3. The correlation between the two determinations in individual patients is often poor (particularly in patients D. R. and A. J.), but in general the ratio of the values approaches unity. Less divergence is found when mean values are considered. For all 28 patients in whom comparisons were made, the mean rate of glomerular plasma flow was 497 cc./min., and the mean rate of tubular plasma flow 466 cc./min.

TABLE III
Glomerulonephritis

Pt.	CIN	Inulin extraction	GPF	CPAH	PAH extraction	TPF	FF	Diagnosis
	cc./min.	per cent	cc./min.	cc./min.	per cent	cc./min.	per cent	
G. W.	67	12.7	528	444	89	499	13.4	Subacute Glomerulonephritis. (Nephrotic Stage)
D. R.	45	12.8	352	519	86	604	7.5	Subacute Glomerulonephritis
A. H.	77	14.1	546	550	90	612	12.4	Acute Glomerulonephritis
W. H.	51	10.2	500	440	79	557	9.2	Acute Exacerbation of Chronic Glomerulonephritis
B. B.	78	9.1	857	592	80	740	10.4	Chronic Glomerulonephritis; Latent Phase
G. I.	83	7.5	1106	696	86	810	10.2	Subacute Glomerulonephritis; Nephrotic Stage
P. M.	85	7.1	1196	932	82	1135	7.5	Chronic Glomerulonephritis; Nephrotic Stage
R. E.	76	13.0	585	373	82	455	16.7	Chronic Glomerulonephritis
B. R.	17	7.6	224	121	50	242	7.0	Chronic Glomerulonephritis
J. M.	56	—	—	293	64	458	12.2	Chronic Glomerulonephritis
M. C.	12	—	—	90	35	257	4.7	Chronic Glomerulonephritis
Mean	56	10.2	614	423	70	545	10.3	

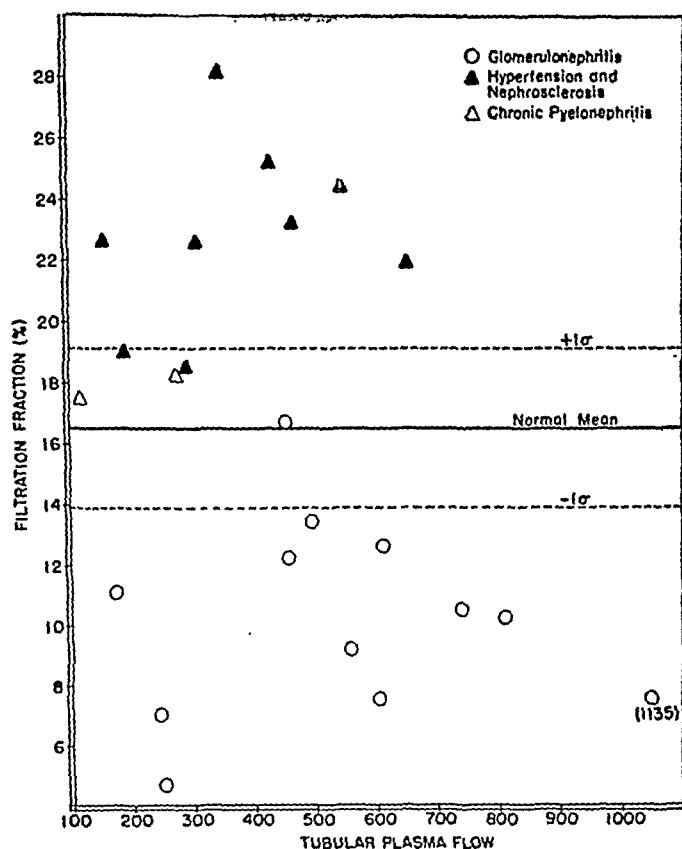


FIG. 1. FILTRATION FRACTION VALUES IN PATIENTS WITH HYPERTENSIVE DISEASE OF KIDNEY AND WITH GLOMERULONEPHRITIS

We consider this convergence of the means to indicate that the individual differences are random and probably not significant, although the heterogeneity of the sample does not permit statistical analysis.

We have found greater fluctuations in the inulin concentrations of successive samples of renal venous blood than in the corresponding arterial samples. This phenomenon is being investigated further, as we feel that it may indicate some intermittency of glomerular filtration.

DISCUSSION

The application of clearance and saturation methods to the study of diseased kidneys has yielded much valuable information. Characteristic functional patterns in various renal disorders have been described (7, 8), involving chiefly alterations in the ratio of glomerular filtration to the mass of actively functioning tubular tissue. Less is known concerning disturbances of renal circulation in abnormal human kidneys, for the reasons set forth in the introduction to this article.

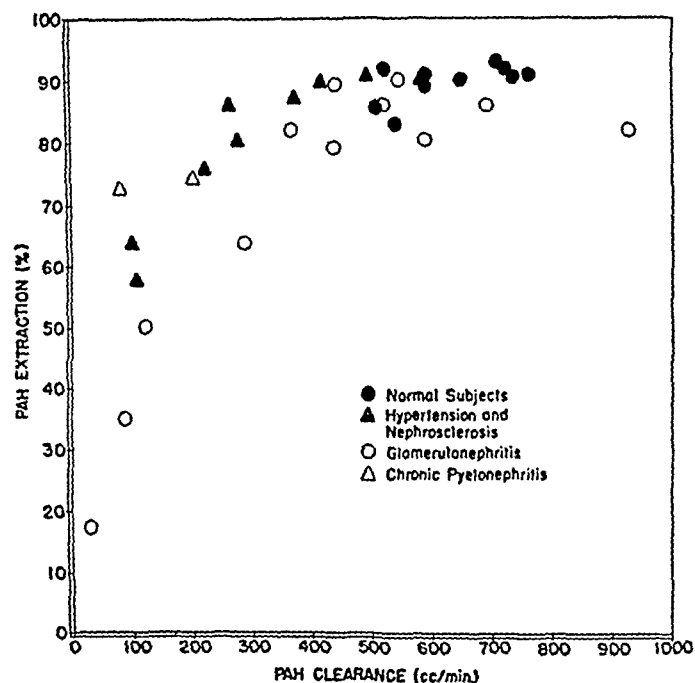


FIG. 2. PAH EXTRACTION AND PAH CLEARANCE IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPERTENSION, GLOMERULONEPHRITIS AND CHRONIC PYELONEPHRITIS

The present investigation has been concerned with the determination of cortical blood flow from the excretion and extraction of inulin and PAH. On theoretical grounds, agreement might be expected in normal subjects between the values ob-

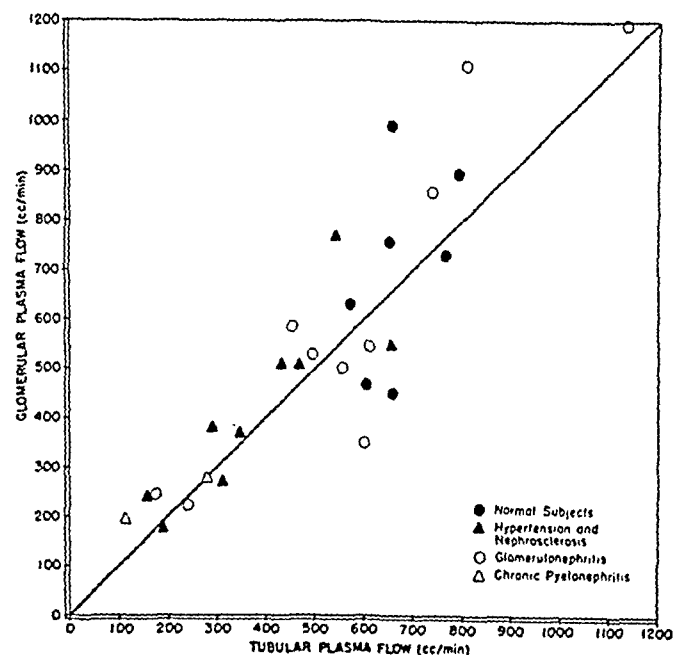


FIG. 3. GLOMERULAR AND TUBULAR PLASMA FLOW DETERMINATIONS IN NORMAL SUBJECTS AND IN PATIENTS WITH HYPERTENSION, GLOMERULONEPHRITIS AND CHRONIC PYELONEPHRITIS

tained by using these two compounds. White and Heinbecker (9) found in the dog that determinations of renal plasma flow from the clearance and extraction of inulin and diodrast differed by less than 10 per cent. Reubi (10), however, has recently claimed that in man estimations of plasma flow from mannitol and PAH clearance and extraction do not agree. Our data show essential agreement between inulin and PAH determinations of plasma flow in seven subjects with normal renal function.

The morphologic studies of Dehoff (11) and of Oliver (12) indicate that an extensive reorganization of the vascular pattern of the cortex takes place in the late stages of both nephrosclerosis and glomerulonephritis. Whereas in the normal kidney the tubules are supplied only with post-glomerular blood, in the diseased kidney numerous channels are to be found through which blood may pass directly from an interlobular artery or afferent arteriole to a peritubular capillary. The development of these vascular short-circuits has been associated by Oliver with the loss of the normal functional predominance of the glomerulus and the appearance of relatively aglomerular tubules. Smith (13) has postulated the existence of "impotent nephrons" lacking tubular activity, through which glomerular filtrate may pass unchanged to collecting ducts.

It is conceivable that an extensive disturbance in the balance between glomerular and tubular blood supply might be reflected in a disproportion between the rates of plasma flow as estimated from inulin and from PAH excretion and extraction (GPF and TPF). We have not found such a disproportion, even in severely diseased kidneys. It is true, of course, that the methods employed yield only an estimate of the total blood supply of all functioning glomeruli and tubules, and it is manifestly impossible to detect from our data any dissociation of flow between the parts of individual nephrons. The results show only that the overall rates of perfusion of glomerular and tubular tissue are similar. This is not necessarily an index of the relative size or spatial relation of these segments. Smith (13) has pointed out that the circulation of interstitial fluid in the kidney makes possible the extraction of diodrast by tubular cells located at a distance from the glomerulus where the filtration of inulin took place. It is probable

that the values we have found in abnormal kidneys represent the means of a variety of rates of flow corresponding to the areas of atrophy and hypertrophy which figure so prominently in microscopic sections of the diseased cortex. The absence of an appreciable difference between rates of plasma flow as determined by inulin and by PAH makes it highly unlikely, however, that the adaptive and regressive changes described by Oliver are associated with any significant redistribution of the blood supply between glomerular and tubular tissue. The only alternative is to assume that in the kidneys under discussion inulin may be excreted by tubular cells or that PAH is excreted entirely by glomerular filtration. The different degrees of clearance and extraction of these substances make either of these possibilities highly unlikely.

Raaschou (14) has pointed out that in diseased kidneys the filtration fraction as calculated from clearances alone is an artificial concept, meaning only the relation between the volume of filtered fluid in the glomeruli and the amount of plasma flowing past active secretory tissue. The same objection might be raised to the use of the ratio of filtration rate to the minimal renal plasma flow as determined from PAH clearance and extraction. Since, however, the filtration fraction so derived equalled the observed extraction of inulin, we feel that it is a valid concept even in diseased kidneys, representing the actual fraction of plasma water filtered at the glomeruli.

A low filtration fraction in the early and moderately advanced stages of glomerulonephritis has been noted previously by other workers (7, 8, 15). We have found that the filtration fraction remains low even in the late stages of this disease. This finding emphasizes the predominantly glomerular localization of the morbid process throughout its course, and suggests that the decreased circulation found late in the disease is secondary to glomerular obliteration. In the acute and subacute stages of glomerulonephritis, particularly in the nephrotic stage, a low filtration rate may be the only functional abnormality noted. A few of these patients actually showed an increased renal blood flow as measured both by inulin and by PAH.

In contrast is the elevation of the filtration fraction in patients with hypertension and hypertensive disease. Here the abnormality is primarily vascu-

lar in origin. Smith and his associates believe that the maintenance of the filtration rate with a decrease in blood flow is due to functional constriction of the efferent glomerular arteriole. Our observations suggest that the filtration fraction tends to fall as the vascular disease progresses. This fall may represent the addition of occlusive changes in the afferent arteriole which counteract the efferent vasoconstriction and lower the intraglomerular pressure.

The recent demonstration by Trueta *et al.* (16) of arterio-venous shunts in the rabbit kidney has given rise to much speculation as to the existence and importance of these shunts in man. If blood is shunted away from the cortex through juxta-medullary channels the extraction of PAH should fall. This has been found to happen in rabbits (17). Our data indicate that in the diverse conditions studied PAH extraction was not decreased until renal damage was moderately advanced, suggesting that the Trueta mechanism is not operative in the early stages of hypertension and glomerulonephritis under resting conditions.

SUMMARY AND CONCLUSIONS

1. Glomerular plasma flow (GPF) has been estimated from the rate of urinary excretion and the arterial-renal-venous inulin difference, and compared with the tubular plasma flow (TPF) obtained from the excretion and renal extraction of sodium para-aminohippurate in normal subjects and in patients with varying degrees of renal impairment.

2. The general agreement between the values obtained for GPF and TPF indicates that there is no extensive dissociation of glomerular and tubular circulation, even in advanced renal disease.

3. The ratio of glomerular filtration rate to renal plasma flow (filtration fraction) is lower than the normal mean in all stages of glomerulonephritis, and higher than normal in nephrosclerosis and chronic pyelonephritis.

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HYPERVOLEMIC ANEMIA IN CIRRHOSIS

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Anemia is usually considered to exist when there is a reduction of hemoglobin, red blood cells or both, per unit volume of blood. This concept is valid in most types of anemia. There is an apparent tendency on the part of the body to compensate for a reduction in circulating red cell mass by an increase in plasma with resultant more or less normal blood volume for the individual (1). We have found this to be true even with the profound reduction of red blood cell mass occurring in cases of aplastic anemia of unknown etiology. There are, however, certain exceptions wherein gross alterations in total blood volume may mask the true hematological state. Reduction in total blood volume in spite of increase in plasma volume has been reported in pernicious anemia during relapse (2-4). Conversely, a significant increase in total blood volume with a relatively greater increase in plasma volume than in circulating red cell mass may, when only routine blood counts are done, suggest a more severe grade of anemia than actually exists. Such cases might be expected to respond poorly to hematinic agents. The following data suggest that the anemia occurring in cirrhosis of the liver is of this character.

METHODS

Hemoglobin determinations were done by the oxy-hemoglobin method on a Klett Summerson photoelectric colorimeter. Normal hemoglobin values for males were considered to be 16 grams per cent, for females 14 grams per cent. The Nesslerization method was employed for plasma protein determinations and readings were made on a Coleman Junior spectrophotometer. Plasma volume was measured by the Evans Blue (T-1824) dye method using a single blood sample as recommended by Gregersen (5). The plasma dye concentration was determined on a Coleman Junior spectrophotometer. The standard curve was made with plasma. Blood for hematocrit determination was drawn from the antecubital vein following release of the tourniquet. In a small series of simultaneous hematocrit determinations on blood drawn in this manner and femoral vein blood there was found to be no essential difference. Heparin was used as an anticoagulant

and hematocrit readings were made after 30 minutes of centrifugation at 3000 r.p.m. Gibson (6) using radioactive iron found the body hematocrit to be about nine-tenths of the large vessel hematocrit. This suggests that hematocrit determinations done in the usual way may be too high. Total blood volume was calculated from plasma volume and hematocrit according to the formula:

$$\frac{\text{Plasma vol. in cc.}}{100 - \text{hematocrit}} \times 100 = \text{Total blood volume}$$

Results of total blood volume determinations done on four healthy young male adults ranging from 174 to 180 cm. in height were found to be 5,051, 5,277, 5,500 and 5,532 cc. These figures are slightly lower than those of Gibson (7) for normal individuals of this height. The calculated normal values for blood and plasma volumes of the patients studied were derived from the chart prepared by Gibson and Evans (7) using the patient's height as the basis of the calculation. It has been suggested that in the presence of obesity or significant weight loss, height or ideal weight be employed to predict normal plasma volume (8).

The figures for hemoglobin and red blood counts corrected for expected normal blood volumes were derived by the following formula:

$$\frac{\text{Patient's hemoglobin}}{\text{Estimated normal blood volume}} \times \text{Blood volume calculated by dye hematocrit method} = \text{Corrected hemoglobin}$$
$$\frac{\text{Patient's red blood count}}{\text{Estimated normal blood volume}} \times \text{Blood volume calculated by dye hematocrit method} = \text{Corrected red blood count}$$

Bone marrow for study was obtained by sternal puncture done under local anesthesia. The relationship between the sternal marrow findings and the peripheral blood counts is depicted on Figure 1.

MATERIAL

Seven cases of cirrhosis of the liver from the Third Medical Division of Bellevue Hospital were studied. The hematological observations on these patients were made prior to and during the administration of therapy for cirrhosis as well as for the anemia which the patients exhibited. The studies were terminated by transfer to another hospital (one case), by death (two cases), and arbitrarily when it became obvious that the patient had not responded to hematinic agents in the expected fashion

HEMOGRAMS AND STERNAL MARROW FINDINGS ON FIVE PATIENTS WITH CIRRHOSIS.						
CASE NO.	II	V	IV	VI	VII	
PATIENT	V.D.	R.G.	A.A.	O.M.	M.F.	
AGE	37	42	49	47	58	
SEX	M	M	M	F	F	
Hb Gm%	13.68	8.95	9.86	7.70	5.25	
RBC	3.68	2.45	3.47	4.68	2.24	
WBC	12,500	13,250	10,950	11,850	9,850	
MCV	92.3	126.5	97.9	66.3	77.6	
MCHb	37	36.5	28.4	16.4	23.4	
MCHbC	40	28.8	29	24	30.1	
STERNAL MARROW						
MYELOBLASTS	0.6%	0.4%	1.5%	1.2%	0.6%	
PREMYELOCYTES	0.6	1.1	1.7		0.2	
NEUTROPHILIC MYELOCYTES	15.3	13.7	11.3	9.0	3.2	
NEUTROPHILIC META MYELOCYTES I	17	19.6	12	12.5	3.8	
NEUTROPHILIC META MYELOCYTES II	17.2	23.1	18.7	19.3	8.0	
NEUTROPHILIC POLYMORPHONUCLEARS	13.3	9.4	18.3	25.3	30.0	
EOSINOPHILS	2.6	4.4	2.0	0.4	1.6	
BASOPHILS	0.5	0.4	0.2	0.2	1.0	
LYMPHOCYTES	9.7	10.2	15.5	22.2	25	
LARGE ATYPICAL LYMPHOCYTES				2.0	2.0	
MONOCYTES				0.3	0.2	
RETICULUM CELLS			1.3			
PLASMA CELLS			1.0		0.4	
ERYTHROBLASTS			2.0		0.2	
MEGALOBLASTS		0.2		0.2		
MACROBLASTS		1.5	1.5		1.6	
NORMOBLASTS	2.2	16.0	13.0	7.4	22.2	
MEGAKARYOCYTES			SCANT	OCC.		

FIG. 1

(three cases). One patient in whom the diagnosis was not clear cut (M. F.) ran an unexpected course.

The data on these cases are summarized in Table I. There are four males and three females ranging from 36 to 58 years of age. All patients but one had histories of excessive alcohol consumption with more than one previous hospital admission for that complaint alone. One male had a history of inadequately treated lues and the female with no history of alcoholism was found to have positive serology on two occasions. The symptoms included weakness of varying duration in all patients and weight loss which was frequently partially masked by ascites formation. Complaints referable to the abdomen were those of vague abdominal discomfort usually promptly followed by the development of swelling. Gastro-intestinal complaints consisted of anorexia in six individuals, bouts of nausea and vomiting in four. Hemorrhagic phenomena had been present at some time in four patients although in the 36 year old male these could be attributed to a concurrent vitamin C deficiency.

Dullness or confusion was present in four patients, milder personality abnormalities in two. Only the non-alcoholic patient showed no signs of mental deterioration. Telangiectasia was present in five cases, hepatomegaly in all, splenomegaly was noticed in only one, jaundice (usually mild or moderate) was present five times, and ascites and edema six times.

Laboratory data are given in the table. Only representative determinations are presented. One patient (M. F.) whose history and physical findings were somewhat atypical was found to have esophageal varices on X-ray as well as biopsy findings compatible with cirrhosis. The diagnosis of cirrhosis was substantiated in two other patients by biopsy studies.

Sternal marrow studies (Figure 1) failed to reveal a megaloblastic bone marrow even in R. G. whose mean corpuscular volume was 126 and who showed a moderately severe grade of anemia. One patient, V. D., who was almost terminal at the time of study manifested a marked depression in the red cell series. No striking abnormalities were noticed, otherwise.

The hematological studies in relationship to blood volume and therapy are presented graphically in Figures 2 to 7 inclusive. No blood volume studies were done on T. M. (Figure 2) but his lack of response to hematinic drugs was characteristic even of patients treated longer and more vigorously. The slight reticulocyte rise observed following institution of therapy was considerably below that which might be expected in an anemia of the degree suggested by his blood counts.

Two patients (V. D. and E. F.) expired shortly after admission to the hospital. It was not possible therefore to observe their response to anti-anemia therapy. However, it will be observed (Figure 3) that the blood counts

TABLE I
Data on seven cases of cirrhosis of the liver

Case	I	II	III	IV	V	VI	VII
Patient	T. M.	V. D.	E. F.	A. A.	R. G.	O. M.	M. F.
Sex	M	M	F	M	M	F	F
Age	36	37	46	49	42	47	58
Ht.		5'6"	5'5"	5'6"	5'10"	5'3"	4'11"
Alcoholism	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.
Lues	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Pos.
Symptoms							
Weakness	3 mos.	Indef.	3 mos.	1½ mos.	Indef.	1 mo.	2 mos.
Weight loss	40 lbs.	Pos.	Pos.	27 lbs.	15 lbs.	Pos.	None
Abdominal discomfort	Absent	Present	Present	Present	Present	Present	Present
Abdominal swelling	None	Present	1 week	Present	2 weeks	Present	2 mos.
Anorexia	Pos.	Pos.	3 mos.	6 mos.	Pos.	1 mo.	None
Nausea	Neg.	Pos.	3 mos.	6 mos.	Pos.	1 mo.	None
Vomiting	Neg.	Pos.	3 mos.	6 mos.	Pos.	1 mo.	None
Bleeding	Ecchymosis gingival	Melena	None	None	Melena hematemesis	Hematemesis	None
Signs							
Confusion	None	Marked	Mod.	Dullness	None	Marked	None
Telangiectasia	Present	Present	Present	Present	Present	Absent	Absent
Hepatomegaly	Present	Present	Present	Present	Present	Present	Present
Splenomegaly			Slight				
Jaundice	Absent	Mod.	Slight	Slight	Mild	Mod.	None
Ascites	None	Present	Present	Marked	Slight	Marked	Marked
Edema	Minimal	Present	Present	Present	None	Present	Mild
Laboratory findings							
Albuminuria	+	+	+	+	+	+	Neg.
Urobilinogenuria		-	1:40	1:20	1:10	1:200	
Bilirubinuria		++++	++++	+	+	+	
Total plasma prot., gm. %	7.6	5.6	5.7	6.5	6.9	6.4	5.6
A/G ratio		23/33	2.7/3.0	2.5/4.0	3.4/3.5	3.0/3.4	2.7/2.9
Cephalin flocculation	Pos.	Str. pos.		Str. pos.	Str. pos.	Str. pos.	Neg.
Thymol turbidity		11		8.6	21.5		
Alkaline phos.	3.4	6.8		5.0	9.1	8.8	12.8
Cholesterol total	267	44	141	67	436	283	252
esters		8	37	49	321	82	116
Non-protein nitrogen		27	57	37	33	25	35
Icterus index		150-240	18	18	21	32	4
Hb. in gm. %	10.1	13.6	11.7	9.8	8.9	7.7	5.2
RBC in millions	3.12	3.68	3.40	3.47	2.45	4.68	2.24
MCV, cu. μ	115	93	103	98	126	68	77
Blood volume in cc.		6506	5365	6147	7043	5290	4425
Plasma volume in cc.		4294	3510	4064	4860	3995	3720
Esophageal varices		Suggestive					Present
Liver biopsy				Cirrhosis	Cirrhosis		Cirrhosis

recalculated on the basis of normal blood volumes for individuals of their height (7) suggested that these patients were not actually anemic.

Intraheptol was ineffective and folic acid produced only an insignificant reticulocytosis in A. A. and R. G. (Figures 4 and 5). The hemoglobin and red blood cell values recalculated on the basis of normal blood volumes are again found to be higher than routine blood counts indicated.

Two patients (O. M. and M. F.) had a microcytic hypochromic type of anemia (Figures 6 and 7). Parenteral liver extract which had been given for the patient's liver disease resulted in no alteration in the blood picture of O. M. However, a reticulocyte response followed by

a rise in hemoglobin (determination made at another hospital following patient's transfer) indicates a favorable response to iron in this case. M. F. likewise showed an improvement in her blood count. She received liver extract and ferrous sulfate simultaneously. It was felt that iron alone would probably have been equally effective.

DISCUSSION

In a study of 132 patients with hepatic disorder Wintrobe (9) concluded that, except when hemorrhage or a complicating infection was associated with it, anemia occurring in liver disease was

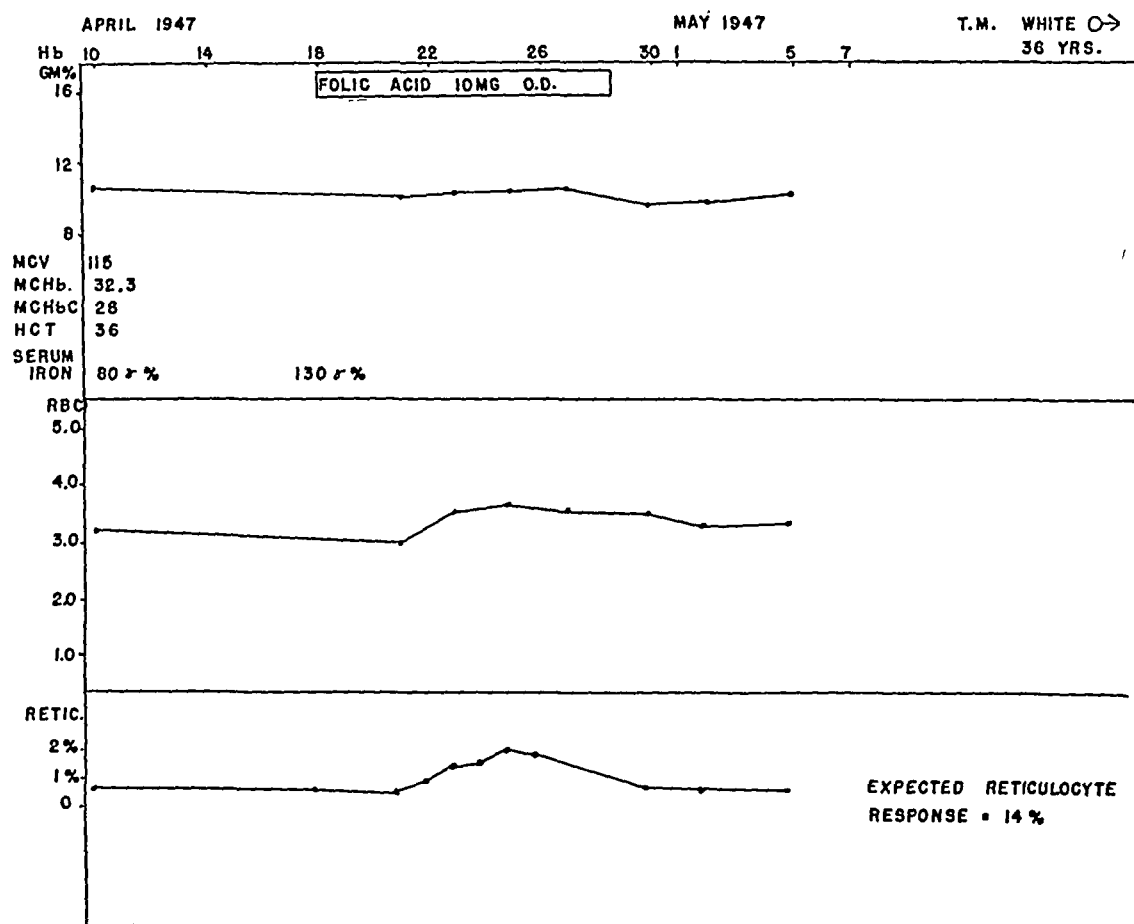


FIG. 2. FOLIC ACID THERAPY IN A CASE OF CIRRHOSIS WITH MINIMAL HEMATOLOGICAL RESPONSE

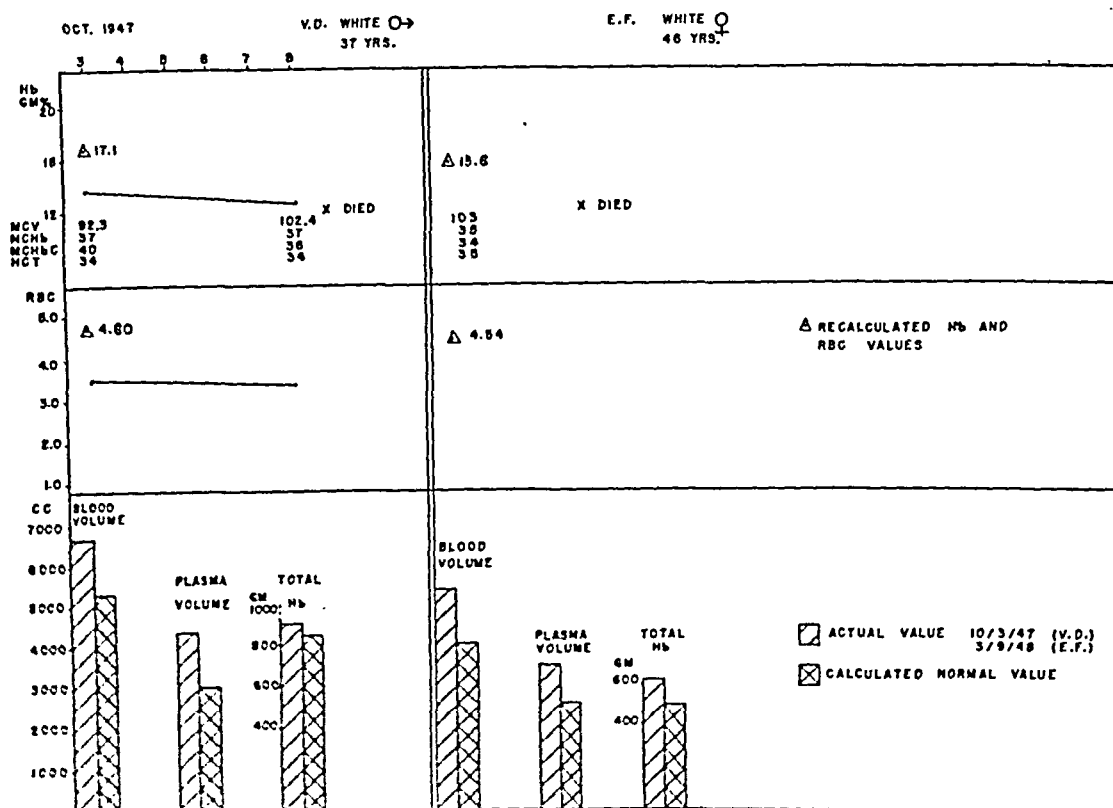


FIG. 3. ACTUAL BLOOD AND PLASMA VOLUMES COMPARED WITH THE CALCULATED EXPECTED NORMAL VALUES IN TWO PATIENTS WHO DIED

Hemoglobin and red blood cell counts recalculated on basis of expected normal blood volumes are shown to be much higher than routine blood counts would indicate.

normocytic or macrocytic. Furthermore when macrocytic anemia was present it was morphologically similar to that seen in pernicious anemia and like the latter manifested spontaneous remissions and was influenced by intramuscular liver therapy. Goldhamer (10) reported a case of cirrhosis of the liver in which free HCl was present in the gastric contents and in which the blood picture simulated that seen in pernicious anemia. There was a response to parenteral liver extract but the figures given suggest that it was very slight. Goldhamer concluded that interference with storage of anti-anemic principle was a factor in the production of macrocytic anemia in cirrhosis. Win-

trobe and Shumacker (11) obtained a clear-cut response to liver therapy in only one out of four patients. Schiff, Rich and Simon (12) prepared extracts from the livers obtained at post mortem from five patients with chronic liver disease, including two with cirrhosis. The extracts were administered to a suitably controlled group of patients with pernicious anemia in relapse. A characteristic reticulocytosis resulted followed by an increase in hemoglobin and red blood cells and marked clinical improvement. This response was attributed to the presence of anti-anemic principle in the liver extract administered.

Hypochromic macrocytic anemia is an infre-

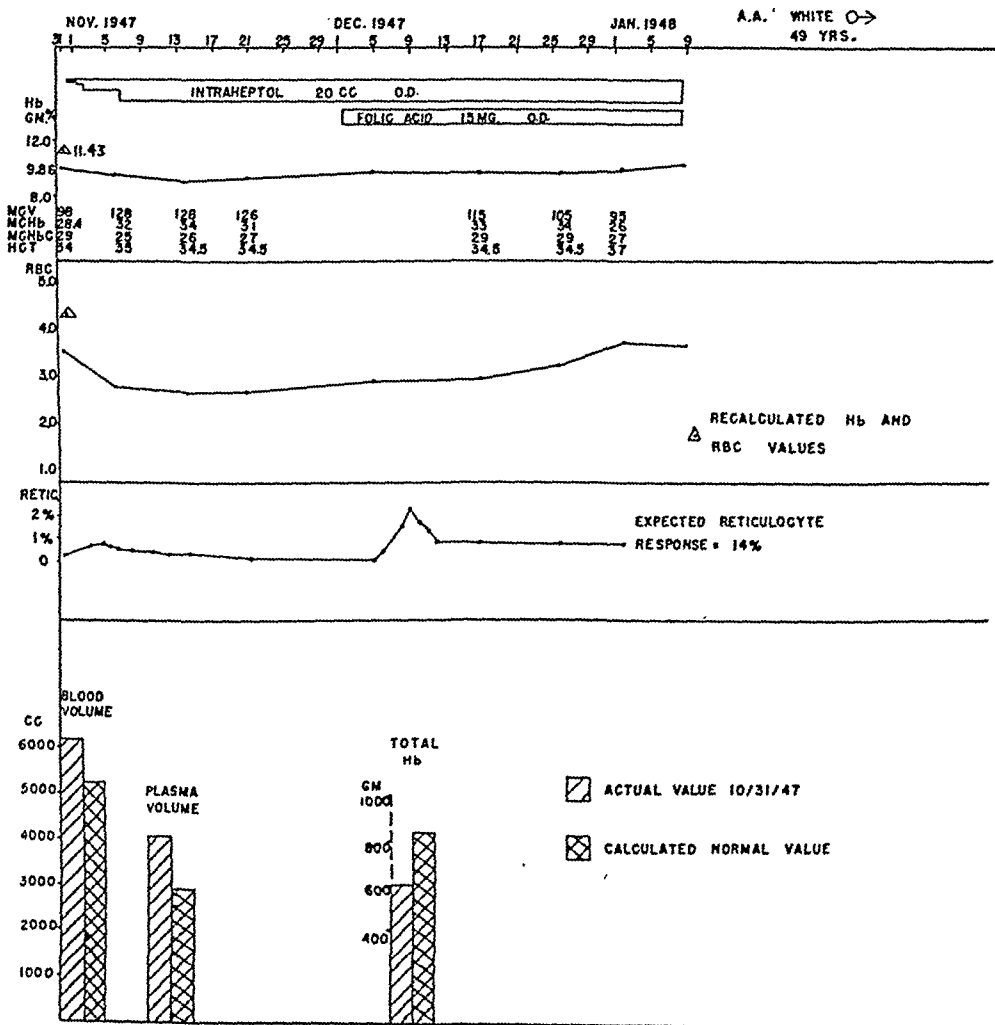


FIG. 4. HEMATOLOGICAL AND BLOOD VOLUME STUDIES IN PATIENT WHO RECEIVED LARGE DOSES OF INTRAHEPTOL AND FOLIC ACID

Hemoglobin and red blood cell values recalculated on basis of expected normal blood volume are seen to be higher than those found on routine counts. Hematological response to therapy poor.

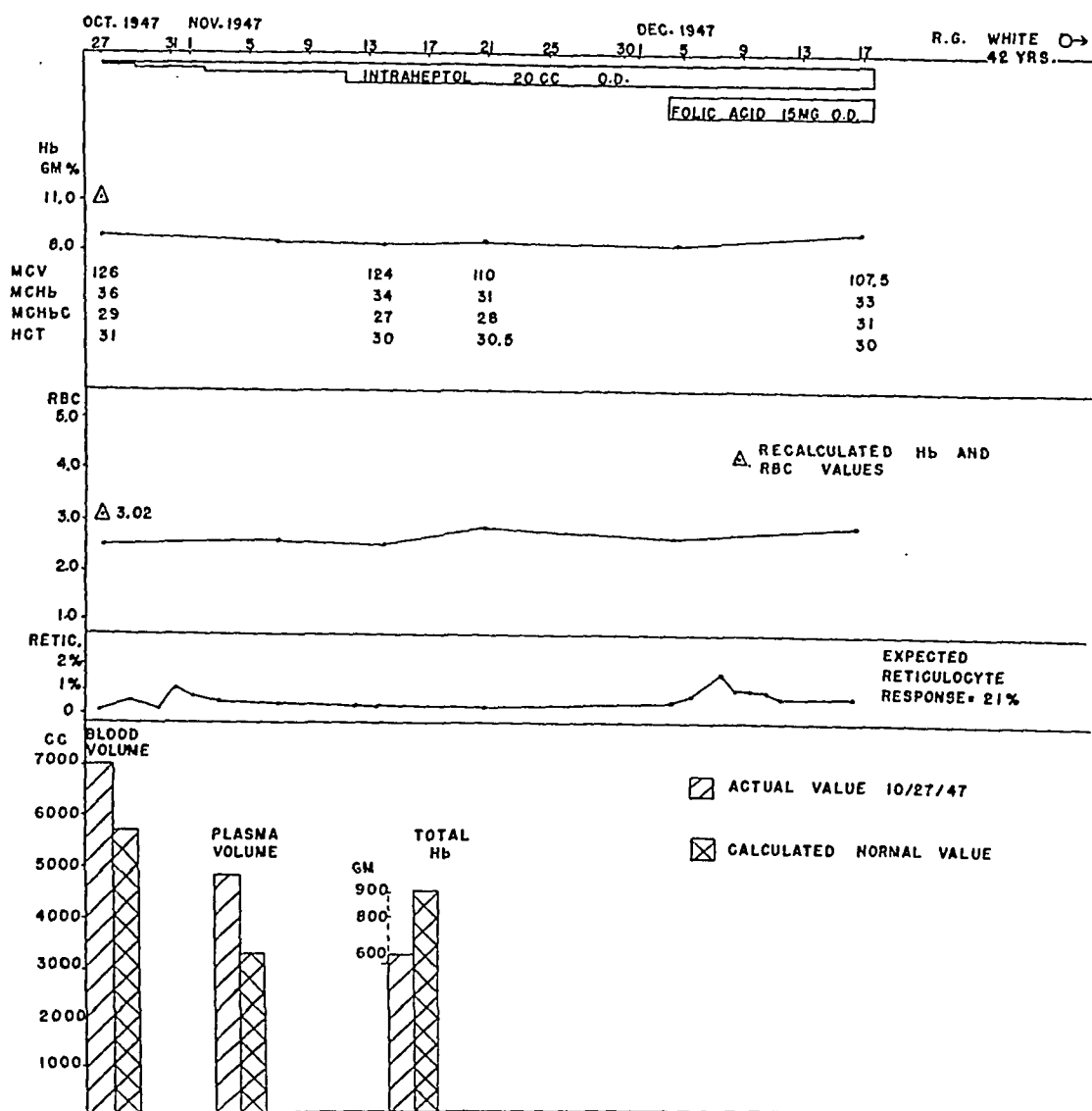


FIG. 5. PROLONGED THERAPY WITH INTRAHEPTOL SUPPLEMENTED BY FOLIC ACID WITH NO APPRECIABLE ALTERATION IN HEMATOLOGICAL PICTURE

Recalculated hemoglobin and red blood cell values 11 grams per cent and 3.02 millions respectively.

quent finding. Davis and Brown (13) describe two cases having mean corpuscular volumes of 100 and 101 cubic microns and mean corpuscular hemoglobin concentrations of 23 and 26 per cent respectively. We had previously noticed the occurrence of this type of anemia in some patients with cirrhosis. In this series of cases three patients (T. M., A. A., R. G.) exhibited a persistently macrocytic hypochromic anemia while in one patient (M. F.) a microcytic hypochromic anemia changed to a macrocytic hypochromic anemia during therapy. The latter finding is probably due to a double deficiency involving depleted iron stores as well as a lack of, or inability to utilize, the erythrocyte maturation factor.

Hypervolemia has been reported as occurring in cirrhosis of the liver (14) as well as in acute liver disease (15). Labby (15) has suggested that failure of the acutely inflamed liver to store blood efficiently may partially explain the concomitant rise in circulating red cell mass found in the latter condition.

Intraheptol is considered to have three units of anti-anemic principle per cc.¹ and would therefore be expected to have an action similar to that of purified liver extract. An extensive literature has recently appeared on the use of folic acid as an effective agent in the treatment of macrocytic anemia of various types (16-24). While the he-

¹ Personal communication from Dr. Elaine P. Ralli.

matological response obtained in pernicious anemia may not be as marked as when parenteral liver extract is used, the results with folic acid are impressive. In sprue and other nutritional macrocytic anemias folic acid therapy compares favorably with liver therapy.

Neither intraheptol nor folic acid altered the hematological status of our patients. The small reticulocyte rise following folic acid administration is considered to be not significant.

When, however, the hematological picture is considered in the light of the marked increase in blood volume it becomes apparent that the majority of these patients actually had either no deficiency in total red cell mass and hemoglobin or only a mild one.

Bleeding phenomena as hematemesis, melena, gingival oozing, and ecchymosis were present or reported in the histories of three male patients. Two of these patients (T. M., R. G.) as well as one (A. A.), whose past history was extremely unreliable, showed a mild hypochromia of the red blood cells with a macrocytosis. Two of the women had a hypochromic microcytic anemia. This is not surprising since mild hypochromic anemia secondary to chronic menstrual blood loss is a common finding. The picture is a little complicated in that both of these patients received liver extract as well as ferrous sulfate. It is interesting to note that the blood picture in M. F. assumed a macrocytic character after her hematological response to therapy. In view of the findings in the

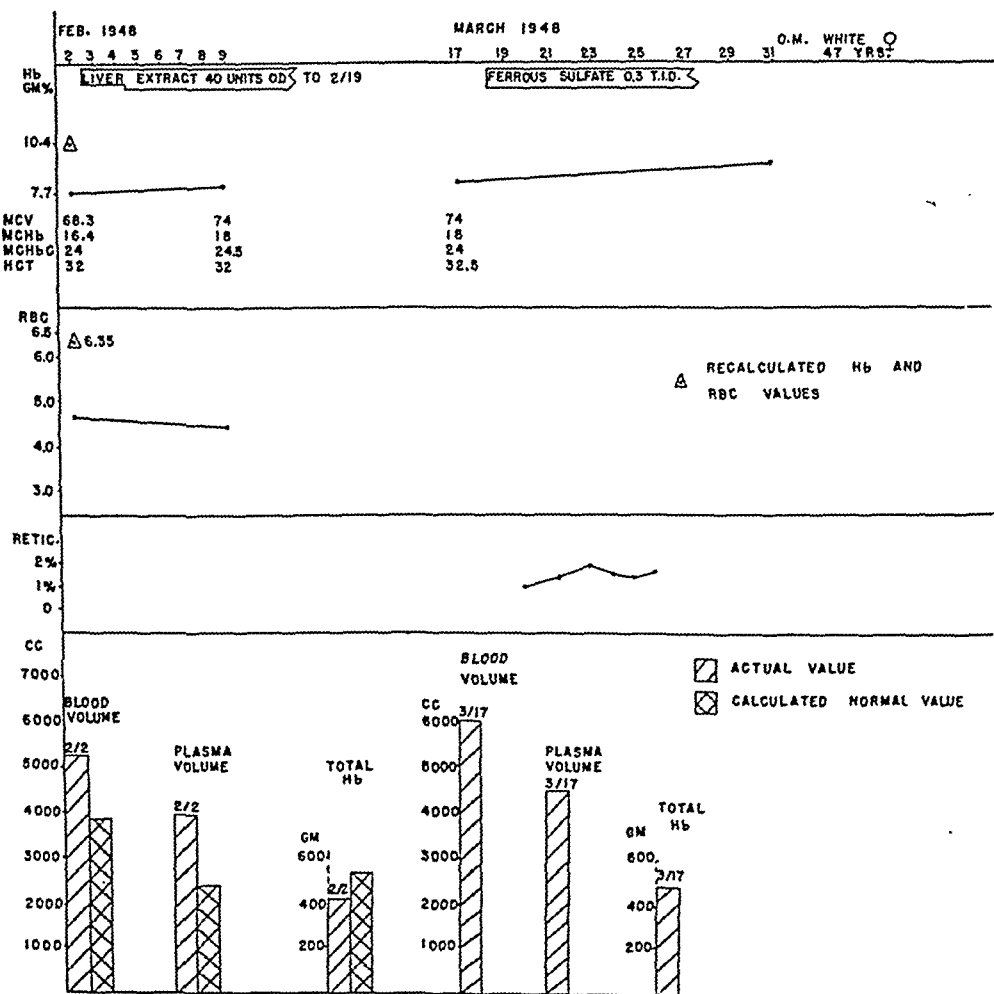


FIG. 6. HYPOCHROMIC MICROCYTIC TYPE OF BLOOD PICTURE SHOWING RESPONSE TO FERROUS SULFATE THERAPY

Blood and plasma volume determinations done on two occasions are markedly above calculated normal values.

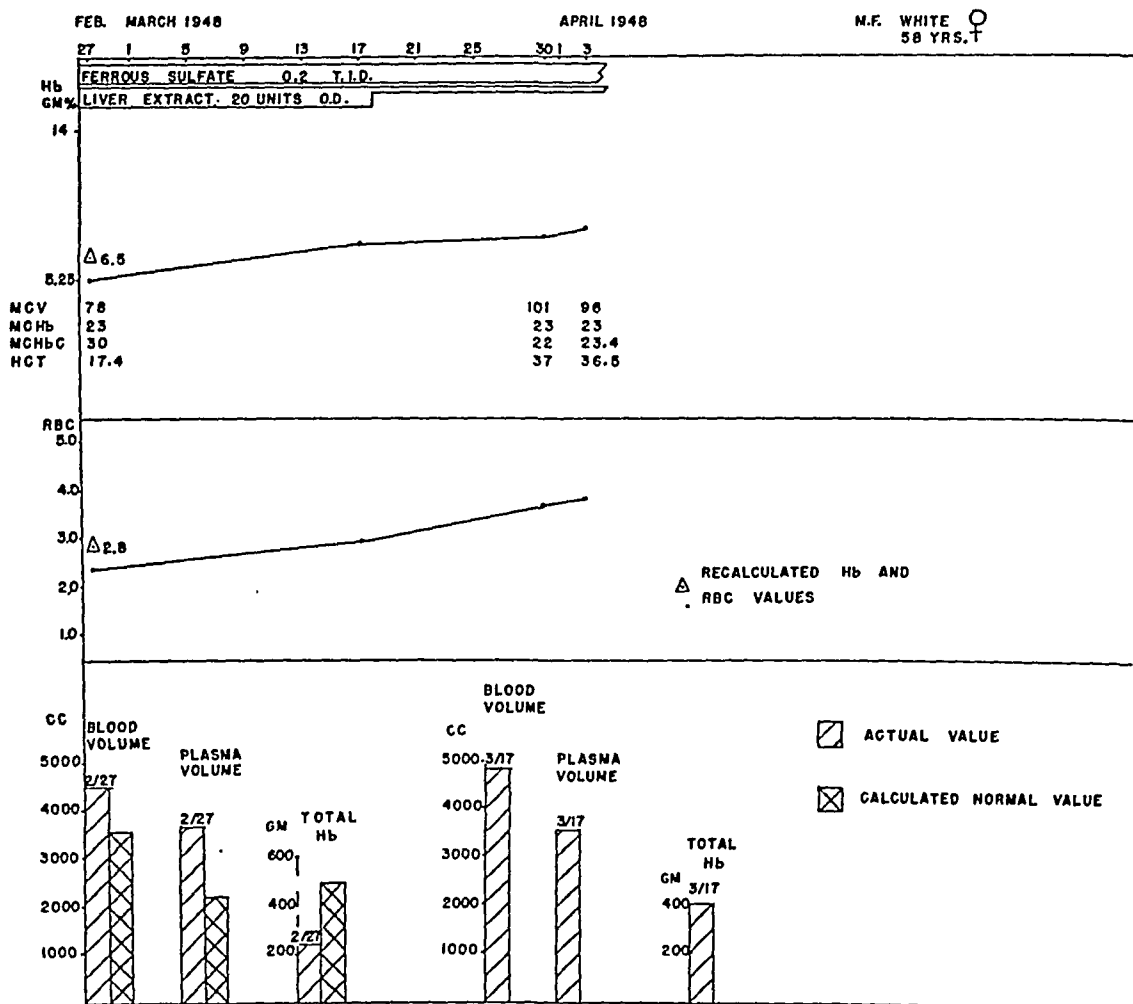


FIG. 7. HEMATOLOGICAL RESPONSE TO LIVER AND IRON THERAPY WITH CHANGE FROM MICROCYTIC TO MACROCYTIC TYPE OF BLOOD PICTURE

other cases reported it is felt that ferrous sulfate alone would have been as effective in these women as when combined with liver extract.

SUMMARY AND CONCLUSIONS

1. The marked increase in total blood volume with a relatively greater increase in plasma than in circulating red cell mass occurring in cirrhosis, when only routine hematological studies are made, suggests a more severe degree of anemia than exists.

2. The presence of an almost normal circulating red cell mass may partially explain the poor response to anti-anemic agents in macrocytic anemia associated with cirrhosis.

3. Hypochromic microcytic anemia occurring in chronic liver disease apparently responds to ferrous sulfate therapy in some cases.

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THE EFFECT OF HYALURONIDASE ON THE ABSORPTION OF PARENTERALLY ADMINISTERED RADIOACTIVE PLASMA PROTEINS IN THE DOG¹

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There are numerous circumstances in which it would be desirable to administer substances subcutaneously, intramuscularly or intraperitoneally that cannot or should not be given intravenously. Since the need for parenterally administered protein and fat is one of the most urgent problems in clinical medicine, we felt it worthwhile to explore the possibility of facilitating their absorption from the tissues by the use of hyaluronidase,² a non-antigenic "spreading factor" first isolated from the testicle (1-3). Sannella (4) observed that this enzyme hastened the absorption of saline solution from the subcutaneous tissues of the rabbit. Hechter (5) found this to be true also in man, not only for saline solution but for plasma as well. Studies were therefore undertaken for a more precise determination of the facts for plasma by the use of plasma tagged with radioactive iodine. Similar observations with respect to fat will be reported (6).

METHOD

Preparation of radioactive iodo-plasma protein

Plasma was iodinated as previously described (7) with the following new modifications. About 2 millicuries of I¹³¹ (as NaI) (half life, eight days)³ were added to 5 mgm. of carrier iodine (as a KI solution) and diluted to 15 cc. with distilled water. One cc. of this solution was diluted 100,000 times with distilled water as standard.

The remaining 14 cc. was mixed with 25 cc. of carbon tetrachloride and to this mixture was added 20 mgm. of potassium iodate and 0.1 cc. of concentrated hydrochloric acid. Free iodine was extracted by the carbon tetra-

chloride, which was separated and added to 500 cc. of plasma (obtained from fresh heparinized dog's blood) containing 20 cc. of 25% sodium carbonate. The violet color of the carbon tetrachloride solution was rapidly discharged on shaking the mixture, which was then allowed to stand for 20 minutes, after which the carbon tetrachloride was separated. The iodinated plasma proteins were then dialyzed in a cellophane bag against cold running tap water for 72 hours to remove unbound ionic iodine. The plasma, now cloudy, was cleared by adding a few grams of sodium chloride and 5-10 cc. of 25% sodium carbonate solution. The volume was measured and 1 cc. was diluted up to 100 cc. and the radioactivity of 1 cc. of this dilution was determined to calculate the per cent of radioactivity incorporated. The pH of the solution was adjusted to 7.4 (glass electrode), with dilute hydrochloric acid. The plasma was then sterilized by Seitz filtration and stored in lead-shielded sterile bottles at 4° C.

Ten to 14% of the radioactive iodine utilized was incorporated in the plasma proteins. The greater the iodine content of iodinated protein, the greater the rate of disappearance from the circulating blood after injection. The iodo-proteins we produced contained about 0.02-0.03% iodine. This amount is well below the upper limit of iodine content required to avoid exceeding the lowest possible degree of denaturation in a protein molecule so tagged. "If one atom of iodine had been incorporated per average protein molecule, the plasma proteins would have contained about 0.2% of iodine, which is the theoretical upper limit of iodine content for obtaining the slowest possible rate of disappearance of a halogenated protein from the circulating plasma" (7), following intravenous injection of iodinated plasma proteins.

Data concerning the stability of the iodine linkage in iodinated plasma protein have been presented previously (7). Examination of a blood specimen, taken 23 days after a dog had been given an injection of radioactive plasma protein subcutaneously, revealed the total absence of radioactivity in the protein free filtrate of the plasma, although considerable radioactivity was present in the plasma. Apparently the radioactive iodine was still bound to the protein.

Animal experiments

Mongrel dogs were given morphine sulfate (2 mgm. per kgm.). Normal oxalated plasma was obtained for preparation of a standard, and the dog then received a

¹ Aided by a grant from the Patrons of Research of the Beth Israel Hospital, Boston.

² The hyaluronidase used in these experiments was obtained as "Hyronase" through the courtesy of Schering Corporation, Bloomfield, New Jersey. The preparation used contained approximately 50 turbidity reducing units per mgm.

³ Obtained from Monsanto Chemical Corporation, Oak Ridge, Tennessee.

TABLE I

*Radioactivity in the circulating blood plasma in per cent of the total radioactivity injected**

Time in hours	Dog 1			Dog 2			Dog 3			Dog 4			Dog 5		
	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	0.3	1.0	50	2.0	1.0	49	1.0	2.0	53	1.0	2.6	63	1.0	0.9	49
3	0.7	1.4	46	3.1	2.0	48	1.5	3.2	46	1.0	2.8	52	1.2	1.6	47
5	0.9	1.8	43	4.1	4.1	46	2.0	4.0	41	1.1	3.5	49	1.3	2.2	45
8	1.5	2.5	37	5.0	14	43	4.5	6.0	38	2.0	6.0	46	1.6	3.9	41
12	2.0	4.0	30	7.1	16	39	11	19	33	4.0	9.5	44	3.8	7.0	36
24	4.0	9.0	25	17	27	28	18	32	23	14	27	32	10	15	28
36	8.3	14	22	19	27	26	19	35	19	19	31	29	14	22	21
48	10	17	20	20	25	24	19	33	16	20	30	24	16	26	21
56	12	18	18	18	24	22	17	22	16	22	29	21	17	24	20
72	11	15	16	17	20	19	13	21	15	18	26	17	14	23	18
96	10	12	12	15	17	17	11	19	12	15	21	13	13	21	16
120	9.0	9.2	11	13	16	14	8.5	15	11	13	19	12	12	19	15
144	8.2	9.2	9.9	12	14	13	7.5	13	10	11	17	11	9.0	17	13
168	7.5	9.0	9.1	10	9.9	14	6.5	12	8.5	9.5	16	10	6.5	15	13
192	6.8	7.2	8.2	9.0	8.0	11	5.9	10	7.5	8.5	14	9.5	6.5	13	12

* About six weeks elapsed between each injection in order to allow the radioactivity in the blood plasma to disappear completely.

subcutaneous injection in the thigh of 10 cc. per kgm. of sterile radioactive plasma proteins. Blood specimens for radioactivity determinations were then taken from the opposite leg at or about the following intervals, in hours: 1, 3, 5, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168. When the radioactivity of the plasma reached zero, usually at the end of about six weeks, the same animal received a subcutaneous injection in the opposite thigh of 12 TRU⁴ of hyaluronidase in 10 cc. of sterile normal saline followed at once by 10 cc. per kgm. of sterile radioactive plasma proteins.⁵ The injection was given slowly and gently into the subcutaneous space and without producing undue tissue pressure. The time required for the mass of subcutaneous fluid to disappear was observed. The thigh injected was immobilized for 12 hours to prevent the introduction of error due to motion or pressure.

When the radioactivity of the plasma again reached zero, the dog was given 10 cc. per kgm. of sterile radioactive plasma protein intravenously in order to observe its rate of disappearance from the blood. For this purpose, samples were drawn at about the same intervals mentioned previously.

⁴ TRU—Turbidity reducing units.

⁵ It has been observed that the spreading response induced by hyaluronidase is influenced by the enzyme concentration and the volume of injection (8). To employ the best conditions for the action of hyaluronidase, 240 µg. of the enzyme or at least 10 times that ordinarily recommended and a volume of plasma protein larger than the potential volume of the injection site were used. The thigh was used because of the tightness of the tissues in this area.

Because of the variation in the rate of absorption from dog to dog, it was found necessary to use the same animal for all three parts of the experiment. Five such experiments were completed. Similar experiments were carried out on two dogs in which radioactive plasma was injected intraperitoneally instead of subcutaneously.

Measurement of radioactivity

Plasma (1 cc.) was evaporated in an aluminum cup to dryness at 37° C. for 24 hours. The radioactivity of this preparation was compared with a standard which was similarly prepared after adding 0.2 cc. of the original radioactive plasma used for the injection to 1 cc. of non-radioactive plasma prepared from the blood sample taken from each dog just before it received the radioactive plasma.

All specimens were measured for radioactivity with a Tracerlab Autoscaler and bell type G.M. tube with mica window (4 mgm. per sq. cm.), shielded with 1.5 inches of lead. All readings were at least five times background. The percentage of injected radioactivity circulating in the blood plasma was calculated by the following formula:

% circulating radioactivity

$$= \frac{\text{activity in counts per minute of 1 cc. of plasma} \times \text{estimated plasma volume} \times 100}{\text{activity of 0.2 cc. of injected radioactive plasma} \times 5 \times \text{dilution factor}^6 \times \text{number cc. injected}}$$

⁶ In some cases it was necessary to dilute the radioactive plasma 100 times before preparing the standard with 0.2 cc. and 1 cc. of non-radioactive plasma.

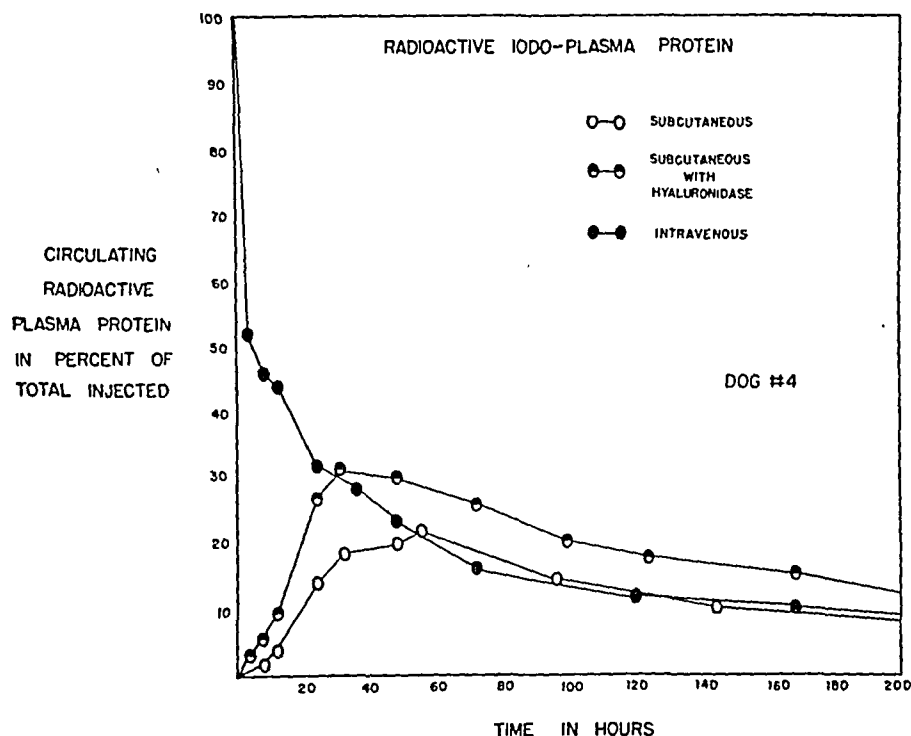


FIG. 1. RATE OF ABSORPTION INTO BLOOD OF INJECTED RADIOACTIVE IODO-PLASMA PROTEIN

The hematocrits of several dogs averaged 50%. Assuming the blood volume to be 10% of the body weight, the plasma volume was estimated to be 5% of the body weight. Table I shows the results of these calculations for all experiments. The results of the three series of observations in each experiment were plotted for comparison. In Figure 1 the disappearance of radioactivity from the plasma after administration by three techniques is shown for one dog.

It will be noted that the disappearance rate of radioactivity after an intravenous injection of radioactive iodo plasma proteins is faster than that observed by Fine and Seligman (7). This may be due to the fact that more denaturation of the plasma protein may have been produced with sodium carbonate treatment or by the use of HCl for the adjustment of the pH to 7.4.

In order to compare the quantity of radioactive plasma, with and without hyaluronidase, absorbed into the circu-

TABLE II

*A comparison of the quantity of circulating radioactivity at various time intervals from zero after the subcutaneous injection of radioactive iodo plasma proteins (with and without hyaluronidase) with the quantity of the circulating radioactivity at similar intervals after the intravenous injection of radioactive iodo plasma proteins in the same dog * †*

Time in hours	Dog 1		Dog 2		Dog 3		Dog 4		Dog 5	
	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase
	%	%	%	%	%	%	%	%	%	%
24	6	12	22	41	17	30	12	25	11	16
48	18	36	40	65	40	62	30	58	29	44
72	28	46	53	74	57	75	47	75	41	63
100	36	56	59	80	65	89	56	88	50	75
120	41	58	69	88	68	95	60	90	53	80

* The data are given in per cent and were calculated by a planimeter determination of the area under the blood plasma concentration curve for each experiment at various time intervals from zero. The area under the curve after the intravenous injection is taken as 100% for each interval. See Figure 1 for an example of these blood curves in experiment 4.

† Several weeks were required between each injection in order to allow the radioactivity in the blood plasma to disappear.

lation from the tissues at any given time with the amount remaining in the circulation after an equal interval following an intravenous injection, the area under each curve for a given time interval was measured by means of a planimeter. The area under the curve of the intravenous injection was taken as 100% for each time interval. Table II presents these data, comparing the results among the various dogs.

RESULTS

The results tabulated in Table I show that in three dogs out of five the effect of hyaluronidase is noted within one hour after the subcutaneous administration of radioactive iodo-plasma proteins. As the concentration levels indicate, the presence of hyaluronidase increases the absorption of the radioactive plasma proteins into the circulation two to three times that without it. Within five hours, similar results are observed in four out of five dogs; within eight hours, similar results are observed in all dogs. Within 36 hours, the rising concentration of circulating radioactivity tapers off. Within 96 hours, the concentration of circulating radioactivity is the same as without the enzyme in two dogs, but is one and one-half to two times that without the enzyme in three dogs.

Similar conclusions are derived from a comparison of the quantity of circulating radioactivity at various time intervals from zero after the subcutaneous injection of radioactive iodo plasma proteins (with and without hyaluronidase) with the quantity of circulating radioactivity at similar intervals after the intravenous injection of the same amount in the same dog (Table II). In 24 hours after subcutaneous injections, the amount of circulating radioactive plasma proteins were twice as much in three, and one and one-half times as much in two dogs receiving hyaluronidase, as compared to five dogs not receiving the enzyme. This difference persists to a somewhat lesser degree for the duration of the experiment. By comparing these quantities of circulating radioactive plasma with that following an intravenous administration, it appears that within 120 hours, 90% of an injection of radioactive plasma protein was absorbed in four out of five dogs receiving hyaluronidase, whereas less than 70 % was absorbed after the same period when no hyaluronidase was given.

It was observed by Hechter (5) that the addition of hyaluronidase to a saline clysis allowed four

TABLE III

*A comparison of the effect of pressure or hyaluronidase on circulating radioactivity at intervals after intravenous or subcutaneous injections **

Dog 2

Time in hours	Subcutaneous injection†	Subcutaneous injection with pressure†	Subcutaneous injection with hyaluronidase†	Intravenous injection†
1	2.0	0.4	1.0	49
3	3.1	0.7	2.0	48
5	4.1	1.5	4.1	46
8	5.0	2.2	14	43
12	7.1	7.0	16	39
24	17	15	27	28
36	19	21	27	26
48	20	26	25	24
56	18	27	24	22
72	17	25	20	19
96	15	23	17	17
120	13	16	16	14
144	12	16	14	13
168	10	13	9.9	14
192	9.0	12	8.0	11

* Six weeks between each experiment.

† Percentage of total radioactivity injected.

and one-half times as much fluid to run into the tissues in a given interval, while a given volume of fluid was taken up 12 times faster. These data do not necessarily reflect accurately the rate of absorption into the circulation. Figure 1 and Tables I and II show that hyaluronidase increases the rate of absorption into the blood of radioactive iodo plasma proteins given subcutaneously, one and one-half to three times. The increased rate for saline presumably would be greater. The increased rate of absorption is an indirect effect of the spreading action of hyaluronidase.

Without hyaluronidase the tension in the dog's thigh at the site of the injected plasma remains high for at least one hour and then diminishes slowly. The fluid mass disappears within four to seven hours. When hyaluronidase is added to the plasma proteins, the fluid mass from the beginning is not tense, but soft, and completely disappears within one hour. As expected, the discomfort due to tension is therefore avoided.

It may be conjectured that pressure may facilitate the spreading out and disappearance of a fluid mass just as hyaluronidase does. The data of such an experiment (Table III) were obtained when a dog turned itself so as to lie on the injected site. It will be observed that the peak of circulating radioactivity was not reached until 48 hours after that observed in a hyaluronidase experiment in the

TABLE IV

Circulating radioactivity after an intraperitoneal injection of radioactive iodo plasma protein

Time in hours	Dog IP. 1*		Dog IP. 2*	
	Intra-peritoneal injection†	Intra-peritoneal injection with hyaluronidase†	Intra-peritoneal injection†	Intra-peritoneal injection with hyaluronidase†
1	1.0	4.0	1.4	2.0
3	10	10	4.0	10
5	22	16	10	19
8	38	23	22	25
12	36	36	26	28
24	32	33	26	26
36	27	30	23	22
48	23	26	21	20
56	22	25	20	18
72	20	22	16	15
96	17	19	13	15
120	15	17	13	12
144	14	15	10	9.5
168	12	13	8.5	9.0

* Six weeks between each injection.

† Percentage of total radioactivity injected.

same dog. The peak level in both experiments was about equal but considerably greater than in the control. Pressure, therefore, by virtue of dissemination, increases the rate of absorption. The rate of increase presumably is related to the degree of pressure applied, but pressure seems to be less efficient than hyaluronidase as a spreading agent.

In three preliminary experiments, which are not included in this series, abscesses developed at the site of injection, probably because unsterile radioactive plasma proteins were used at that time. The repeated injection of hyaluronidase into these areas did not seem to increase the extent of infection.

Inasmuch as hyaluronidase is considered to be a protein-like substance, it might be expected to be antigenic. The repeated injection of 240 μ g. in all dogs used in these experiments resulted in no evidence of anaphylaxis.

In two experiments, hyaluronidase did not increase the rate of absorption of radioactive iodo plasma proteins administered intraperitoneally (Table IV). This is consistent with the assumption that hyaluronidase acts by depolymerizing the intercellular cement substance.

CONCLUSIONS

1. Hyaluronidase increases the rate of absorption of plasma proteins injected subcutaneously, one and one-half to three times.
2. It is not antigenic in the doses used herein.
3. It acts more efficiently than pressure.
4. It does not increase the rate of absorption of plasma proteins administered intraperitoneally.

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THE ACCELERATION OF LINEAR FLOW IN THE DEEP VEINS OF THE LOWER EXTREMITY OF MAN BY LOCAL COMPRESSION¹

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INTRODUCTION

With the increasing age and life expectancy of the population (1) and the higher incidence of thromboembolic disease in older age groups (2-4) the problems of phlebothrombosis are becoming increasingly urgent. Particularly needed is more information concerning the etiology and pathologic physiology of venous thrombosis, so that adequate measures may be taken to prevent its consequences. The present communication presents the results of experiments upon one of the factors recognized as important in the formation of intravenous clots, namely, the velocity of venous flow.

In 1856, Virchow postulated that alterations of the blood current, of the blood chemistry and of the blood vessel wall, are factors important in the etiology of venous thrombosis (5). Since then numerous papers have been devoted to alterations of the vessel wall and of the blood chemistry and morphology but only scant and sporadic attention has been paid to changes in the venous current. It has been shown by careful pathological and roentgenographic studies that the deep venous system of the calf is the most important site of development of phlebothrombosis leading to pulmonary embolism (6-10). It has also been suspected that slowing of the venous current contributes to this situation (11-14). However, as yet, no one has related a slowing of flow to anatomic abnormalities in the deep veins of the leg nor has the effect of compression on the velocity of flow been previously reported. In this study particular attention was paid to the general form of the deep veins of the calf as well as to the velocity of flow and the effects of local compression.

MATERIAL AND METHODS

The subjects were a group of 12 male and eight female patients from the ward and private services of the Massa-

chusetts Memorial Hospitals. Their ages ranged from 17 to 70 years. All subjects were studied after resting for 45 minutes in a quiet room the temperature of which was maintained relatively constant ($\pm 2^\circ \text{C}$).

With the patient supine upon the X-ray table the entire lower extremity from the instep to the upper third of the thigh was encased in a series of loosely fitting blood pressure cuffs or in an inflatable legging (of a G-suit). The heel was elevated so that the calf was free of the table and the leg was immobilized by properly applied sandbags at the foot.

A 20 gauge, $1\frac{1}{2}$ -inch needle was inserted through pre-cainized skin into a distal vein on the dorsum of the foot in such a fashion that the tip of the needle was well within the pressurized area when the G-suit or cuffs were inflated. To the needle was attached a three-way stopcock and tubing leading to an infusion of normal saline solution which was allowed to flow at a constant slow rate for the purpose of keeping the needle patent between injections. All injections of test substances were made directly into the vein through the side arm of the three-way stopcock. After control observations were completed the limb was pressurized by inflating the garment or blood pressure cuffs at pressures of 20 or 35 mm. Hg, whereupon the test procedure was repeated.

In two patients an additional 18 gauge, two-inch needle was placed in the femoral vein just proximal to the upper margin of the inflatable garment. To this a five-inch segment of an 18 F nylon catheter was attached so that blood samples could be collected in oxalated tubes every four seconds. Three cc. of 0.5% Evans Blue Dye (T-1824) were injected into the foot vein. Blood samples were then collected from the femoral vein for periods of 50 to 100 seconds. This procedure was carried out before and during the application of pressure as described above. The blood samples were centrifuged at 3,000 R.P.M. for 30 minutes and the supernatant plasma after appropriate dilution was analyzed for dye concentration using the Coleman photoelectric colorimeter. Thus, the appearance, maximum concentration, and disappearance of the dye in the femoral vein were accurately timed.

The test substances used in carrying out these experiments were solutions of 35% Diodrast, 20% Decholin sodium,² and 0.5% Evans Blue Dye (T-1824).³ Diodrast was not given to patients with a history of allergy or previous diodrast reactions, and decholin was not administered in the presence or history of jaundice or liver

² Generously supplied by Mr. Charles Miles, Ames, Co., Elkhart, Indiana.

³ W. G. Warner Co., New York, New York.

¹ This investigation was supported in part by the Squibb Institute for Medical Research, New Brunswick, New Jersey.

disease. All injections were made by the same person who carefully timed the rate of injection. When discrepancies in the rate of injection occurred, the test was discarded or repeated. Stopwatches were calibrated to one-tenth of a second.

RESULTS

A. Appearance time

In the early phases of this investigation two independent observers noted through a fluoroscopic screen the time required for a 4 cc. dose of diodrast to pass from the needle to a lead marker applied to the upper third of the calf. The time from the start of injection until the dye reached the marker was noted and termed the appearance time. When the control observations were completed the limb was pressurized at 20 m. Hg and then the procedure repeated.

Fifteen control observations were made in eight cases before the application of pressure and were compared with 11 observations made during pressurization of the limb at 20 mm. Hg. The control mean appearance time was 21.78 seconds as contrasted with a mean appearance time of 11.38 seconds during pressurization (Table I).

B. Linear velocity of flow

Because the method reported above was open to certain criticisms and also because in some instances the deep veins of the calf were poorly visualized during fluoroscopy, it seemed desirable to confirm these results, if possible by more accurate methods. To this end lead markers were fixed at specific intervals on calf and thigh and the distance

between the markers carefully measured, so that the linear velocity in centimeters per second could be determined.

After observing fluoroscopically the appearance time of the diodrast in the calf and allowing 15 minutes for all the injected dye to be cleared from the veins, the dye was again administered and serial roentgenograms of the calf and thigh taken at exactly timed intervals during the period that diodrast would be expected to be in the field under study. Another pause was allowed for clearing, and the limb was then pressurized at 20 mm. Hg. Five minutes later the test was repeated and roentgenograms taken at exactly the same intervals as before. In three patients simultaneous control observations were made on the opposite extremity subjected to the same procedures as the experimental limb except that it was never pressurized.

As might be expected, technical difficulties including the exacting team work required, proper positioning of the limb, the absolute necessity for immobility of the subjects, and limitations imposed by the amount of X-ray to which any one patient might be safely exposed, prevented this technique from yielding results that could be accurately interpreted in every experiment. As a matter of fact, in several experiments a marked increase in velocity during pressurization resulted in the dye having already left the field by the time the thigh film was taken during pressurization. However, in no experiment could the results be interpreted as indicating that pressurization had decreased the velocity of venous flow. On the contrary, in practically every experiment, particularly when the

TABLE I
Effect of pressurization on the appearance time of 35% diodrast in the calf as visualized by fluoroscopy

Patient	Age	Before pressurization			During pressurization			Per cent decrease in mean appearance time during pressurization
		Number of tests	Range	Mean	Number of tests	Range	Mean	
J. C.	21	2	56-59	57.5	2	27-30	23.5	59.1
Z. I.	52	2	28-30	29	1		21.8	24.8
T. A.	40	2	30-32	31	2	16-18	17	45.1
I. V.	48	1		22	1		10	54.5
B. U.	73	3	10-16.2	12.06	1		6.2	48.4
G. U.	17	2	12-12.2	12.1	1		6.0	49.6
M. A.	45	1		6.5	1		2.5	61.5
C. O.	32	2	4.2-4.0	4.1	2	4.6-3.5	4.05	1
Mean				21.78			11.38	43.0



FIG. 1

Unretouched photographs of X-ray films taken to show the progress of an injected dose of Diodrast (4 cc.) before (1 and 2) and during (3 and 4) the application of 20 mm. Hg pressure to the limb. Films 1 and 3 were taken 10 seconds, films 2 and 4, 30 seconds after injection. Dye may be seen just entering the distal femoral vein in film 2, while it has reached at least to the external iliac vein in film 4.

major portion of the diodrast entered the deep venous system of the leg, the velocity of flow was unquestionably increased, in some instances to a striking degree.

Figure 1 illustrates unretouched photographs of serial roentgenograms of the dye in the deep veins of the calf and thigh of a 48-year-old female before and during the application of 20 mm. Hg pressure. The calf films were taken 10 seconds and the thigh films 30 seconds after the injection. It is obvious that the injected diodrast moved at a markedly increased velocity when the limb was pressurized.

The results of such studies in five cases suitable for analysis are recorded in Table II. In each instance the application of pressure resulted in a definite increase in the linear velocity as determined by this technique. The mean increase in four cases was 45.4 per cent and the range 22 to 75 per cent while in the fifth case linear velocity was increased by 131 and 170 per cent in two tests.

Simultaneous observations in the control limb showed insignificant changes as compared with the

TABLE II

Effect of pressurization on the linear velocity of venous flow as measured from serial roentgenograms

Patient	Age	Linear velocity, cm./sec.		Per cent increase in linear velocity during pressurization
		Before pressurization	During pressurization	
B. U.	73	1.09	1.33	22
S. T.	42	1.4	3.8	170
L. Y.	48	1.8	4.16	131
J. P.	44	1.2	1.8	50
M. E.	55	4.5	5.7	49
		1.6	2.1	31
		2.0	3.5	75

pressurized limb, while repetition of the test in the experimental limb in two of five cases after the release of pressure revealed the velocity of flow to have returned to pre-pressurization values.

C. Circulation times

1. Foot-to-tongue

Additional corroborative evidence of the acceleration of venous flow during the application of

TABLE III

Effect of pressurization on the foot-to-tongue circulation time as measured by decholin sodium

Patient	Age	Circulation time (sec.)		Per cent decrease in circulation time during pressurization
		Control	During pressurization	
T. A.	24	37	18.2	51
J. M.	64	38.4	32	17
R. H.	72	27.5	17.5	36
S. M.	69	48.5	35.7	26
H. E.	67	87	62	29
H. U.	33	49	41	16

pressure was obtained by foot-to-tongue (Decholin) circulation times in six cases. The circulation times in all instances during pressurization were decreased. The percentage decrease ranged from 16 to 51 with a mean of 29 per cent (Table III).

2. The foot-to-femoral vein circulation time as measured by Evans Blue Dye (T-1824).

The limb venous circulation time was estimated in two cases before and during the application of pressure, by measuring the concentration of injected dye in consecutive four-second samples from the femoral vein. In both cases the appearance time of the dye was markedly decreased during the application of pressure.

Figure 2 illustrates the results of this procedure in a 44-year-old male. It is noteworthy that the dye before pressurization was not present in the femoral vein until 20 seconds after injection at the

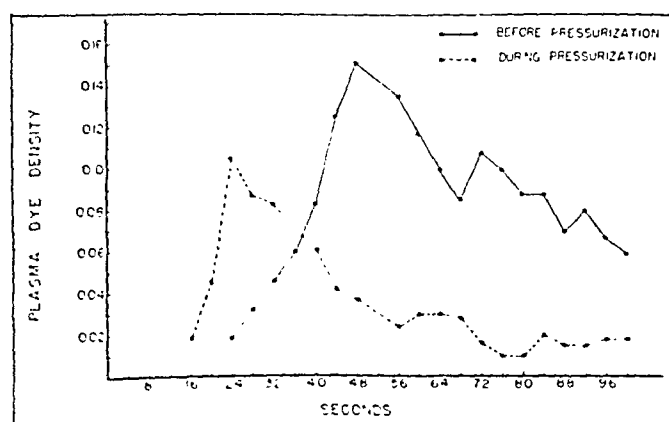


FIG. 2

Illustrates the effect of pressurization of the extremity on the appearance time and concentration curve of Evans Blue Dye (T-1824) in plasma samples obtained by continuous sampling from the femoral vein.

foot whereas during pressurization it was present in 12 seconds. In addition, the shape of the concentration curves indicated that the veins were cleared of the injected dye in less time during the application of pressure. These findings indicate the velocity of venous flow was increased as measured by this technique.

Additional observations

In the course of this investigation the following collateral observations were made upon the size and shape of the leg veins and are reported because of their probable influence upon velocity of venous flow.

(a) In younger individuals the deep veins of the calf were noted usually to be narrow and straight as delineated by the contrast medium, which moved along rapidly in them. In older individuals, particularly those over 40, the deep veins not infrequently exhibited saccular dilations in which the diodrast tended to puddle, often remaining as long as 15 minutes.

(b) Veins well filled with diodrast as viewed in serial roentgenograms frequently exhibited a measurable decrease in diameter during the period of local compression along with the concomitant increase in linear velocity of flow.

(c) The application of pressure usually did not alter the distribution of the diodrast between the superficial and deep venous systems of the extremity, but rather accelerated the flow in both.

(d) Increasing the pressure from 20 to 35 mm. Hg did not cause a perceptible further increase in the velocity of venous flow.

(e) In six patients the velocity of venous flow as judged from the foot-to-tongue and foot-to-pharynx circulation times was similarly accelerated whether the source of local compression was an inflatable legging, an elastic stocking or a carefully applied elastic bandage.

(f) When diodrast was well visualized in the veins of the leg during pressurization it was usually present for a shorter total time than during the control period. This finding and the concentration curves of Evans Blue in femoral vein blood both suggest that the "venous emptying time" (13) was decreased by the application of pressure.

DISCUSSION

It is recognized that large changes in the skin temperature of the digits may alter the circulation time, as demonstrated by Smith, Allen and Craig (11) and Stead and Kunkel (15). It is also obvious that a certain velocity is imparted to the test substances in the process of injection. In the present study diligent efforts were therefore made to control both factors. Furthermore, collateral studies of foot-to-tongue circulation times and simultaneous skin temperatures of the toes failed to reveal more than slight changes in the temperatures when the velocity of venous flow was increased during mild pressurization (16). The magnitude and uniformity of the velocity changes *observed strongly suggest that the linear velocity of venous flow in the leg veins is increased by the local application of pressure.*

Ochsner has recommended compression of the limbs by elastic bandages as a prophylactic measure against phlebothrombosis and pulmonary embolism in surgical patients, on the supposition that compression of the superficial veins would drive more blood through the deep vessels and thus accelerate flow in these channels (17). In the present study the application of pressures of 20 to 35 mm. Hg seldom altered the distribution of the injected diodrast in the deep and superficial veins but did accelerate the flow in both.

It has been reported previously from this laboratory that the local application of pressures of as little as 30 mm. Hg decreases the *volume* of blood flow into the limbs (18). The present experiments demonstrate that in spite of the decrease in volume of arterial blood flow into the limbs there is simultaneously during mild pressurization an increase in linear velocity of venous flow from the limbs. The observation that the veins appear smaller during compression suggests that the observed acceleratory effect is due primarily to a disproportionately greater decrease in cross-sectional area of the venous bed than the decrease in the volume of blood flow into the limbs during such compression. It is significant that marked increases in the linear velocity of venous flow could be produced by the local application of pressures of as little as 20 mm. Hg pressure. Since this pressure (in contrast to higher pressures) causes only slight decrease in inflow (18), it is

probably near the optimum pressure for the maximum acceleratory effect. The possible therapeutic applications of this principle are now under study in this clinic.

SUMMARY AND CONCLUSIONS

1. The application of local pressure of 20 to 35 mm. Hg to the lower extremity of human subjects increased the velocity of venous flow—as measured by fluoroscopy, serial roentgenograms, foot-to-tongue circulation times, and limb venous circulation times.

2. Pressures of 20 mm. Hg applied to the lower extremities of man significantly increased the linear velocity of flow in the deep veins, but did *not alter significantly the distribution of injected diodrast between the superficial and deep venous channels.*

3. It is suggested that the mechanism of the increase in linear velocity of venous flow during local compression is the concomitant decrease in the total cross-sectional area of the venous beds.

The authors are genuinely indebted to Doctors George Levene and Harry Lerner and to Miss Anne Lally and the X-ray technicians of the Department of Radiology of the Massachusetts Memorial Hospitals for cooperation and aid in carrying out the radiographic techniques employed in this study.

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THE EFFECT OF EXERCISE AND BODY POSITION ON THE VENOUS PRESSURE AT THE ANKLE IN PATIENTS HAVING VENOUS VALVULAR DEFECTS¹

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The purpose of this investigation was to determine directly and record continuously the effects of body position and of walking on the venous pressure at the ankle in patients having incompetent veins of the lower extremity. Smirk (1), in 1936, reported the first direct measurement of venous pressure at the ankle during walking. His measurements were made on one normal subject. Beecher, Field and Krogh (2), in the same year, reported on the indirect measurement of the venous pressure in the human leg during walking. Others (3-7) have reported the effects of exercise and position on the venous pressure in the leg measured both by direct and by indirect methods. Recently Pollack and Wood (8, 9), and Henry (10) have given preliminary reports of direct studies of venous pressure in the human leg during walking.

In the present study the venous pressure was recorded photographically by means of an electrical strain-gauge manometer (11).³ The manometer was attached to the subject's lower extremity by means of a specially constructed aluminum support that was taped to the lateral surface of the leg. Care was taken that no tape was placed over the vein to be used for measurement of the pressures. The manometer was coupled to this aluminum support with two double-ended universal clamps (Figure 1).

Polythene tubing 15 cm. long with an internal diameter of approximately 0.5 mm. was used as a venous catheter.⁴ A venipuncture was performed with a number 17 needle in the great saphenous vein as it passes anterior to the medial malleolus. The catheter was passed through the

needle into the vein for a distance of about 10 cm.; then the needle was withdrawn, leaving the catheter in place. The manometer system was flushed frequently with heparinized saline solution.

Respiration was recorded by means of a thermocouple mounted in a plastic nose piece. The heart rate and the electrocardiogram were measured through a Sturm and Wood cardiometer (12).

The venous pressure was recorded continuously in the supine, sitting and resting standing positions. After these maneuvers the patient walked on a horizontal power-driven treadmill at 1.7 m.p.h. for periods ranging from 15 to 60 seconds.

The vertical distance between the tip of the catheter and the third thoracic interspace was measured in the sitting and standing positions. The hydrostatic pressure exerted by a column of blood extending over this distance was calculated, 1.06 and 13.55 being used as the specific gravity of blood and mercury respectively. The zero base lines in the sitting and standing positions were determined by recording the pressure when the surface of a column of fluid in a tube (1 cm. internal diameter) connected to the catheter was held on the surface of the leg level with the position which had been occupied by the catheter tip in the lying, sitting and standing positions. This procedure was necessary in order to avoid errors due to capillarity of the catheter.

RESULTS

In the supine position the average venous pressure in seven patients with primary varicose veins⁵ was 7.2 mm. of mercury with a range from 5.7 to 8.5 mm. In six patients with incompetent superficial (greater saphenous) veins associated with a history of previous iliofemoral thrombophlebitis⁶ the average venous pressure was 10.7 mm. with a range from 8.3 to 14 mm. of mercury in the supine position. In the sitting position the average venous pressure at the ankle in ten patients with

⁵ This term has been used to indicate uncomplicated varicosities of the greater saphenous system.

⁶ These patients clinically appeared to have chronic venous insufficiency of the deep veins in addition to varicosities of the superficial system.

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² Fellows in Medicine, Mayo Foundation.

³ Manufactured by Statham Laboratories, Los Angeles, Calif.

⁴ Manufactured by Surprenant Electrical Insulation Co., Boston 7, Massachusetts.



FIG. 1. STRAIN-GAUGE MANOMETER ATTACHED TO THE ANKLE

incompetent veins of the lower extremity was 52 mm. of mercury with a range from 31 to 61 mm. The average venous pressure at the ankle in the resting standing position was 81.9 mm. of mercury with a range from 63 to 97 mm. (Table I).

In the sitting position, the average difference between the measured venous pressure and the calculated hydrostatic pressure of a column of blood extending from the third thoracic interspace to the ankle was 0.3 mm. of mercury. In the standing position, this difference was 0.6 mm. (Table I). It is evident that while the patient is at rest in the sitting or standing position the venous pressure at the ankle is sufficient to support a column of blood approximately up to the third thoracic interspace, the level of the middle of the right atrium (13).

In seven patients who had primary varicose veins, walking at 1.7 m.p.h. uniformly produced a decrease in the mean venous pressure at the ankle averaging 37.4 mm. of mercury with a range from 29.5 to 47.4 mm., which was maintained for the

duration of the 30-second to 60-second period of walking (Table II). In six patients who had incompetent greater saphenous veins and a history of previous iliofemoral thrombophlebitis, walking produced an average fall of only 11.1 mm. of mercury in the mean venous pressure at the ankle (Table III). The decrease in venous pressure at the ankle in normal subjects produced by walking 1.7 m.p.h. averaged 64 and ranged from 54 to 71 mm. of mercury.

In the patients who had uncomplicated greater saphenous vein incompetency the average time required for the venous pressure to return to the resting standing level after the walk was completed was 2.8 (1.2 to 5.5) seconds as contrasted to the 31 (8 to 57) seconds required in normal subjects (9) (Figure 2a and b).

In the normal subjects after the initial one or two steps of walking the maximal venous pressures generated during subsequent steps of the walk were considerably less than the control resting standing pressure (Figure 2a) (9). In patients who had

TABLE I

Comparison of venous and hydrostatic pressures at the ankle in patients who had incompetent veins of the lower extremity when in the seated and standing positions

Patient	Measured venous pressure, mm. of mercury	Calculated hydrostatic pressure, mm. of mercury
	Seated	Seated
1	60	58
2	61	61
3	31	34
5	50	50
6	50	51
7	54	54
8	52	53
9	55	55
10	55	55
Average	52	52.3
Normal subjects*	58.8 (55-64)	60.2 (55-66)
Patient	Standing	Standing
1	86	87
2	89	89
3	63	63
4	97	97
5	71	72
6	82	84
7	84	84
8	82	82
9	83	85
10	84	84
11	80	80
Average	81.9	82.5
Normal subjects*	90.5 (88-97)	92.5 (88-100)

* Average and extreme values obtained in five normal subjects (9).

uncomplicated valvular defects of the greater saphenous system, the maximal pressure exerted during the stable period approached and frequently equaled the resting standing pressure (Figure 2b). In the patients who had incompetency of the greater saphenous vein and a previous iliofemoral thrombophlebitis the maximal pressure during each step usually exceeded the hydrostatic pressure throughout the period of walking. In these patients the maximal venous pressure during the steps within the stable pressure period of the walk averaged 29 mm. of mercury greater than the resting standing value (Figure 2c). After walking, the venous pressure at the ankle in normal subjects and patients who had uncomplicated greater saphenous incompetency returned to the resting

TABLE II

Venous pressure at the ankle in patients who had simple greater saphenous vein incompetency during quiet standing and while walking at 1.7 m.p.h.

Patient	Mean venous pressure, mm. of mercury		
	Quiet standing (A)	Walking 1.7 m.p.h. (B)	Decrease in pressure (A-B)
1	71	34	37
2	81.9	34.5	47.4
3	84.3	45	39.3
4	81	41	40
5	85	49.6	35.4
6	85.5	56	29.5
7	79	46	33
Average	81.1	43.7	37.4
Normal subjects*	86.6 (79-92)	22.3 (11-31)	64.3 (54-71)

* Average and extreme values obtained in 11 normal subjects (9).

standing control level, while in those patients who had greater saphenous incompetency as well as a previous iliofemoral thrombophlebitis the pressure increased to an average of 3 mm. of mercury higher than the resting standing control level within one second after the completion of the walk and then gradually decreased within a period of 60 seconds to the original control pressure.

COMMENT

In the resting sitting and standing positions in normal subjects as well as in patients who have venous insufficiency the venous pressure at the ankle is equal to the hydrostatic pressure of a col-

TABLE III

Venous pressure at the ankle in patients who had greater saphenous vein incompetency and a history of iliofemoral thrombophlebitis, during quiet standing and while walking 1.7 m.p.h.

Patient	Mean venous pressure, mm. of mercury			
	Quiet standing, start (A)	Quiet standing, end	Walking 1.7 m.p.h. (B)	Decrease in pressure (A-B)
8	86	87	69	17
9	86	89	73.5	12.5
10	62	64	47	15
11	96	104	85	11
12	102	102	96	6
13	94	99	89	5
Average	87.7	90.8	76.6	11.1

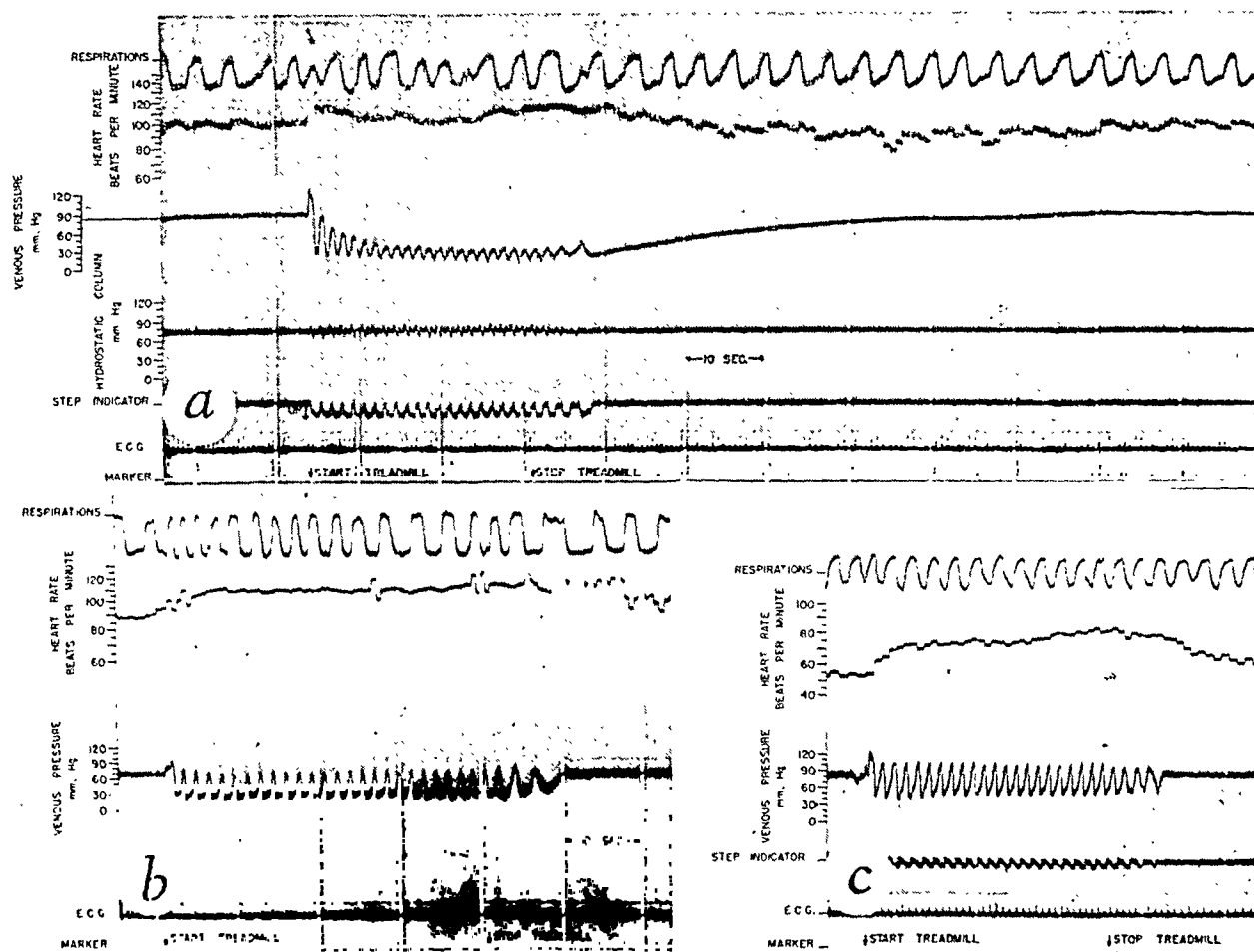


FIG. 2. EFFECT OF WALKING 1.7 M.P.H. ON THE VENOUS PRESSURE AT THE ANKLE

a. Normal subject. *b.* Patient who had primary varicose veins. *c.* Patient who had incompetent greater saphenous vein and a history of iliofemoral thrombophlebitis.

umn of blood up to the level of the right atrium (1, 5-10). The decrease of venous pressure at the ankle in normal subjects produced by walking on a horizontal treadmill is due to the pumping action of the muscles and the competency of the venous valves in preventing reflux of blood into the emptied segments of the veins during the period of muscular relaxation (9). In patients who have uncomplicated primary varicose veins, the valves are unable to prevent the return flow of blood during muscular relaxation and the venous pressure returns relatively quickly to the hydrostatic level, thus accounting for the higher mean pressure during the walk and rapid return to the hydrostatic control level after the walk has been completed.

Interpretation of the genesis of the venous pressure tracings obtained from patients who have incompetent great saphenous veins and a history of previous iliofemoral thrombophlebitis is un-

certain. At first it would appear that there was interference with the outflow of venous blood from the leg in this condition. The higher resting standing pressure at the end of the walk than at the beginning, the higher mean pressure during the stable period, and the marked rise above hydrostatic levels during each step of the stable walking period as contrasted to those obtained from patients having primary varicose veins point to this explanation. However, the fact that no significant difference was found between the extremes of pressures of the individual steps during walking in patients who had primary varicose veins and in patients who had incompetency of the superficial system and a history of iliofemoral thrombophlebitis does not support this interpretation. Even with a critically damped manometer system the effects of acceleration generated by the motions of walking on the manometer and hydrostatic systems are difficult to control. Therefore, the sig-

nificance of the contours and extremes of the pressure variations recorded during walking is difficult to assess. The use of mean pressures, as determined by planimetry, is much less subject to criticisms of this type.

The fact that in four patients suffering from cardiac disease, who had elevated resting standing venous pressure at the ankle due to cardiac causes, the venous pressure at the ankle decreased during walking to the same range as in normal subjects indicates that the venous valves and the action of voluntary muscles have more to do with the regulation of venous pressure in the leg than does central venous pressure (14).

It is not inconceivable that determinations of venous pressure at the ankle during exercise may be used as a diagnostic aid, and as a means of assaying the efficacy of surgical treatment of abnormalities in the venous systems of the leg.

SUMMARY AND CONCLUSIONS

Venous pressure in the greater saphenous vein at the ankle was studied in 13 patients with varicose veins while resting in the recumbent, seated and standing positions and while walking on a treadmill.

Confirmatory data were obtained indicating that the venous pressure at the ankle in the resting, sitting or standing positions in normal subjects and patients with incompetency of the saphenous veins is sufficient to support a column of blood to approximately the level of the third thoracic interspace.

Walking on a level treadmill at 1.7 miles per hour produced an average decrease in mean venous pressure at the ankle of 37 (30 to 47) mm. of mercury in seven patients with primary varicose veins and 11 (5 to 13) mm. of mercury in six patients with varicose veins associated with a history of a previous iliofemoral thrombophlebitis. In normal subjects this value was 64 (54 to 71) mm. of mercury.

In patients who had uncomplicated incompetency of the greater saphenous vein the average time required for the venous pressure to return to the resting standing level after cessation of walking was 2.8 (1.2 to 5.5) seconds. In those patients with a history of previous iliofemoral thrombophlebitis the pressure rose to levels slightly in excess of the control resting value

within one second after completion of the walk as contrasted to the 31 (eight to 57) seconds required for the pressure to return to the control values after walking in normal subjects.

These data are in harmony with the concept that the function of the venous valves in conjunction with the action of voluntary muscles is of primary importance in the regulation of venous pressure at the ankle.

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THE INHIBITION OF FROSTBITE WHEELS BY THE IONTOPHORESIS OF ANTIHISTAMINIC AGENTS¹

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Skin whealing associated with localized frostbite is a well recognized phenomenon and has been described in detail by Lewis and his co-workers (1, 2). Although no positive evidence of histamine liberation in frostbite has been advanced, it has been postulated that histamine or a histamine-like substance is involved in the formation of these lesions (2). Recently, Frommel and Picquet have reported beneficial results from the antihistaminic drug, dimethylaminoethyl-ethylbenzylaniline hydrochloride (Antergan), in the treatment of frostbite in guinea pigs (3).

The inhibition of histamine wheals by the oral or intradermal administration of beta-dimethylaminoethyl benzhydryl ether (Benadryl, Parke-Davis) and N'pyridil-N'benzyl-N'dimethylethylenediamine hydrochloride (Pyribenzamine, Ciba) has been demonstrated conclusively (4-6). A similar inhibition can be achieved locally by the iontophoresis of Pyribenzamine (7).

In an effort to gain additional information regarding the role of histamine in the pathogenesis of local frostbite, we have studied experimentally produced frostbite lesions before and after the iontophoretic administration of Benadryl and Pyribenzamine. The results obtained are presented in this paper.

METHODS

Twelve healthy young males were used as subjects. The ventral surfaces of the forearms and/or thighs were shaved. By means of an ordinary iontophoresis apparatus, various test solutions were introduced into rectangular areas of skin measuring 5 × 5 cm. The positive electrode

was constructed of copper screening covered with flannel. The flannel was saturated with the solution to be tested and was secured firmly to the skin by means of a canvas strap. A current density of 0.5 milliamperes per sq. cm. was applied for four minutes in each case. The solutions introduced by electrophoretic means were (1) 0.9% NaCl, (2) a saturated solution of Pyribenzamine hydrochloride, and (3) a saturated solution of Benadryl hydrochloride. Since each subject acted as his own control, the various solutions were usually applied to the same extremity in rectangular areas one inch apart. In those cases in which sufficient surface area was not available, Pyribenzamine and a saline control were placed on the skin of one extremity, while Benadryl and a saline control were applied in a similar manner to the skin of the opposite extremity. After one hour the erythema which routinely followed the iontophoresis of any of the solutions had usually disappeared. Localized frostbite was then produced by means of a jet of cold air directed at the skin. The air was first dried and was then passed through coils of copper tubing embedded in an insulated cask of dry ice. The rate of flow was measured by means of a standard gasometer placed in the system, and the temperature of the air was determined by means of a thermocouple placed at the end of a 16-inch length of 3/8-inch rubber tubing which served as the outlet. Although the intensity and duration of the stimulus varied somewhat from subject to subject, the same stimulus was always applied to the areas tested on any particular subject during a single experiment. It was found that a jet of air at a temperature of -40° F. applied for ten seconds resulted in satisfactory and reproducible lesions. In a single experiment the same cold stimulus was applied to each of the treated areas and to one untreated area nearby. Observations were made of the time of onset, degree and size of wheal formation.

RESULTS

During, and for a short time following, the iontophoresis of any of the substances used, the subjects reported slight discomfort, usually described as "burning or tingling" at the site of application. This discomfort was equally great whether saline, Benadryl, or Pyribenzamine was used. Similarly, no differences between the saline control and the anti-histaminic agents were noted with regard to local erythema or papule formation.

¹ This work has been performed in collaboration with the Medical Department, United States Army, which has assigned Medical Corps Officers to the Quartermaster Climatic Research Laboratory for research on adaptations of man to environmental stresses.

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The time required for freezing the treated and the untreated areas did not vary significantly. Onset of freezing was heralded by a pricking or burning sensation and blanching of the skin. Within a few seconds, frost began to accumulate on the frozen skin and after ten seconds or more (depending on the intensity of the stimulus), a hard, frozen, cream-colored area, approximately 1.5 cm. in diameter, could be picked up between the fingers. This thawed in a matter of 60 seconds. Within a few minutes the frostbitten area became red and was sharply demarcated from the surrounding skin. In the untreated areas, a wheal invariably began to form within five minutes after removal of the stimulus, continued to swell until it reached a maximum in approximately 20 minutes, and gradually subsided in from one to four hours.

The wheals were graded I to III:

- I = Minimal or no wheal formation
- II = Slight to moderate wheal formation
- III = Marked wheal formation

The whealing was strikingly inhibited in the areas where the histamine antagonists had been introduced (Table I, Figure 1). Benadryl was slightly less effective than Pyribenzamine. Iontophoresis of normal saline had essentially no effect on wheal formation. When wheals did appear in the areas treated with the anti-histaminic agents, the time of appearance was invariably delayed. In most cases the duration and intensity of the cold stimulus were not sufficient to produce vesicle formation. In the few subjects in whom vesicles were produced they tended to be less severe in the areas treated with Benadryl or Pyribenzamine. In all instances the frostbitten skin was tender and



FIG. 1. THE INHIBITION OF FROSTBITE WHEEL ON THE THIGH BY PREVIOUS IONTOPHORESIS OF PYRIBENZAMINE HYDROCHLORIDE

S.C. = saline control, P. = pyribenzamine. Cold stimulus applied to both areas was jet of air at -40° F. for ten seconds.

somewhat painful for approximately 48 hours. The redness persisted for several days and was followed by scaling with eventual disappearance of the lesion in approximately three weeks. A comparison of the treated and untreated areas revealed no significant differences in the progress and ultimate outcome of the frostbite lesions.

SUMMARY AND CONCLUSIONS

1. Introduction of the histamine antagonists, Pyribenzamine hydrochloride and Benadryl hydrochloride, by an iontophoretic technique inhibited the formation of frostbite wheals in the human skin.

2. In these experiments no other effects of anti-histamine agents on the type or progress of localized frostbite lesions were observed.

3. It is concluded from these studies that histamine or a histamine-like substance is liberated in the human skin in the early stages of frostbite.

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TABLE I

The effects of antihistaminic agents on frostbite wheals in twelve subjects

Area frostbitten	Number of subjects showing various degrees of wheal formation		
	Grade I	Grade II	Grade III
Untreated	0	1	11
Saline Iontophoresis	0	2	10
Benadryl Iontophoresis	10	1	1
Pyribenzamine Iontophoresis	11	1	0

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THE CONSTRUCTION OF NORMAL STANDARDS FOR CARDIAC OUTPUT IN MAN

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INTRODUCTORY AND THEORETICAL

The present method of calculating normal cardiac output standards in man involves a fallacy, and has led in practice to erroneous conclusions (1). This is a serious matter for clinicians, since it has been shown by Starr that the first indications of symptomless heart disease may be given by abnormalities in cardiac output (2). Thus it is reasonable to suppose that in the next few decades the determination of cardiac output will become as common as that of basal metabolism, or more so. Consequently it is the object of this paper to present new standards, based on more adequate statistical methods. Use has been made of the data already in the literature, of a new series of 50 subjects so far only reported in abstract (3), and of partly published data on 174 normal individuals collected by Starr and his co-workers.

The introductory and theoretical part of this paper refers to the construction of cardiac output standards irrespective of the method used for measuring output. The main standards developed thereafter refer to the Starr ballistocardiograph; in this connection a new constant for use in the Starr formula for stroke volume is given. Lastly, some biometric relations between the various methods are examined, and as a result tentative recommendations with regard to cardiac catheterization data are made. There are at present insufficient catheterization data available on normal subjects from which to decide whether the same standards may be used as for the ballistocardiograph. In any case the ballistocardiograph seems the more appropriate instrument to use in determining whether a person is slightly abnormal or not.

Material

The material used in this analysis comes from three sources. Firstly, Table I reports measurements made personally with the ballistocardiograph on 50 healthy young men, when I was working in Dr. Starr's laboratory in 1943. In this study classification of physique by Sheldon's method (4) was undertaken and the somatotypes of the subjects are given in the last column; I am indebted to Dr. W. C. Dupertuis for help in this aspect of the work. Secondly, the raw data of the extensive ballistocardiographic series of 200 healthy persons reported by Starr and Schroeder in 1940 (5) have been available to me. This series is of particular value because the subjects have been followed for eight to ten years (2) and those now suffering from any cardiovascular disease have been eliminated from the present calculations, leaving 174 persons. Thirdly, in the last section of the paper, use has been made of all the substantial and detailed series of cardiac output data in the literature, including results obtained by catheterization, acetylene and ethyl iodide methods.

The reliability of cardiac output determinations and the relations between results obtained by different methods

Two points must be considered before undertaking a detailed and thus laborious biometric analysis of cardiac output data. First of all, it is clearly useless to construct standards for a physiological function which changes greatly from minute to minute or day to day in an unpredictable manner. We must first enquire what is the *reliability*²—the repeatability—of the measurement.

² *Reliability* must of course be clearly distinguished from validity. Reliability refers to how repeatable the measurement in question is, and validity to whether it in fact measures what it purports to measure. Clearly any

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TABLE I

Ballistocardiograph cardiac output data for 50 healthy young men

Subject number	Age	Height	Weight	Blood press.	Mouth temp.	Heart rate	Stroke volume	Cardiac output	Somatotype		
	<i>years</i>	<i>cms.</i>	<i>kgs.</i>				<i>cc.</i>	<i>l/min.</i>			
1	20	179.3	68.6	113/70	98.8	68	100	6.80	3	3	4
2	20	191.4	67.4	98/72	98.4	74	100	7.40	2½	2	6
3	20	167.6	66.9	124/69	98.0	63	99	6.24	3	5	2
4	21	177.6	63.2	118/78	98.2	62	99	6.14	3½	3	4½
5	21	172.7	79.1	113/85	98.0	58	109	6.32	5½	4	1½
6	21	162.8	66.5	99/73	97.5	52	101	5.25	5	4½	1
7	21	181.8	76.9	107/64	97.7	70	93	6.51	4	4½	3
8	21	182.6	62.3	122/76	98.7	58	126	7.31	2½	2	5½
9	21	183.3	63.2	103/58	98.3	59	98	5.78	2	3	5
10	22	182.5	67.4	107/73	98.4	57	112	6.39	2	3½	4½
11	22	186.6	71.9	118/76	98.2	49	108	5.29	3	3½	4½
12	22	189.0	71.9	117/80	98.5	59	95	5.61	2½	3	5
13	22	183.0	88.6	120/77	97.8	57	115	6.55	5	4½	1½
14	22	178.2	90.9	114/84	97.3	55	94	5.17	5	5	1
15	22	183.2	68.1	122/77	98.4	65	103	6.70	2½	3½	4½
16	22	184.3	66.0	110/75	99.0	59	100	5.90	2	3	5
17	22	167.7	61.0	102/67	97.8	55	109	6.00	4	4½	3
18	22	184.4	80.5	105/74	98.0	58	112	6.50	2½	5	3
19	22	168.7	72.4	110/79	98.2	60	93	5.58	5½	3	2
20	22	166.4	70.5	106/73	98.5	64	101	6.46	5	3½	2
21	22	180.4	76.0	107/76	97.9	51	109	5.56	4	4	2½
22	22	183.3	77.8	104/71	97.9	52	117	6.08	4½	3	4½
23	22	177.7	77.8	118/81	98.7	75	107	8.03	3	5½	2
24	23	165.5	66.5	109/70	98.7	69	105	7.25	4½	4½	2
25	23	168.8	56.4	103/72	98.0	64	93	5.95	3½	3½	4
26	23	175.8	76.9	96/68	97.6	55	103	5.67	3½	5	2½
27	23	175.8	59.1	111/69	98.0	58	92	5.34	2½	3	5
28	23	173.1	66.0	114/75	98.1	65	93	6.04	3	5½	2½
29	23	186.2	75.5	98/72	97.5	56	93	5.21	3	3	4½
30	23	181.0	74.6	103/74	98.6	74	104	7.70	4	4	2½
31	23	173.6	66.9	97/68	97.6	64	88	5.63	2½	5	3
32	23	175.2	76.5	108/71	97.5	61	96	5.85	4	5	2
33	23	177.1	72.4	109/70	98.4	68	95	6.46	4	5	3
34	23	167.1	62.8	112/78	98.9	78	91	7.10	3	5½	2
35	24	189.7	81.4	101/70	98.2	66	114	7.53	2½	4½	4
36	24	178.0	67.4	114/73	98.4	83	90	7.47	3	3½	4½
37	24	176.2	77.4	104/70	98.5	73	91	6.64	6	2½	2
38	24	183.3	70.5	105/72	98.1	65	111	7.22	3	3½	4
39	24	173.6	77.4	111/75	97.8	55	90	4.95	5½	3½	2
40	24	178.0	69.1	104/65	98.0	51	99	5.05	4	3	4
41	24	172.6	75.5	100/63	97.8	50	112	5.60	2½	6	1½
42	24	172.3	61.9	111/77	99.1	76	95	7.72	3	3½	4
43	24	189.7	79.6	113/73	98.6	57	119	6.78	2	4	4
44	25	177.0	68.6	101/68	98.0	53	103	5.46	3½	3½	4
45	25	167.8	68.6	106/67	98.1	53	102	5.41	5	2½	3
46	25	173.0	62.8	108/81	98.4	68	80	5.44	3½	4	4
47	25	168.5	62.3	101/75	97.4	46	111	5.11	4	3½	3
48	26	163.4	48.2	97/65	98.1	64	85	5.44	2	4	4
49	26	177.8	70.5	113/67	98.6	58	112	6.50	2	6	2½
50	27	182.8	61.9	113/68	98.5	54	121	6.54	1½	2½	6
Mean	22.8	177.2	70.3	108.4/72.4	98.17	61.3	102	6.21	3.4	3.9	3.3
Standard Deviation	1.5	7.05	7.9	7.0/3.1	0.44	8.2	9.8	0.79	1.1	1.0	1.3

This point must be emphasized simply because, at least in its statistical aspects, it has been thoroughly neglected by physiologists and clinicians, who often instead assume their measurements to measurement which is not reasonably reliable cannot be valid, but the converse does not hold.

be a great deal more reliable than they really are. The simplest way of quantitating reliability is by giving the correlation coefficient between two determinations, the second taken a few minutes or days after the first. This test-retest correlation is known as the *coefficient of reliability*. Despite

its importance, it has only been reported for one cardiac output method, the Starr ballistocardiograph; various more or less vague statements are all that we have to go on for other techniques. In estimations by the ballistocardiograph the reliability obtained from two tests seven minutes apart on subjects well rested, so that variation of the cardiac output with the passing of time is minimised, is 0.91 (3). The data given by Starr *et al.* (6) lead to a very similar figure for the ethyl iodide method. The statement by Grollman (7) that a retest nearly always falls within 10% of the initial value indicates that a similar figure would be a reasonable approximation for the acetylene method; while the catheterization method would seem to be slightly less reliable, from the statements of Nickerson *et al.* (8) and Warren *et al.* (9).

Though this reliability is certainly high enough to make the construction of standards worth while, it is not nearly so high as for physical measurements, or indeed as for the impressionistic assessment of physique by a skilled observer. This brings us to the second point. Can a single standard be constructed which applies to all methods of measuring cardiac output, in particular to the ballistocardiogram and to the catheterization methods? He would be a bold man who asserted unequivocally that such a standard is possible and can be constructed with present data. But he would be rash, also, who maintained one method's standards have no bearing on another's; for the quantitative relations between two methods are a subject for biometrical analysis, and arguments from the superior validity of one particular method, however justified, are not relevant to this particular problem.

The published results show an astonishing agreement between the different methods under most circumstances, when due regard is paid to the relatively low reliability of each method. The point is this: method A *cannot* correlate any better with method B than it does with itself, and probably correlates with B a good deal less. When comparing methods A and B, however, it is possible by statistical means to neutralize the unreliability of the tests, and see how closely the two methods would agree if they were not subject to such troubles: to find the true correlation of the phenomena measured, were the methods of meas-

urement not fallible. This is known statistically as correcting the coefficient for attenuation, and is done simply by dividing the method A-method B correlation by the square roots of each method's reliability coefficient (10). The Starr ballistocardiograph stroke volume for 25 subjects, covering wide age and surface area ranges, correlated with the ethyl iodide stroke volume to the extent of 0.86 (6). When corrected for attenuation so as to represent the true correlation between methods when the unreliability is taken away, this figure

becomes $\frac{0.86}{\sqrt{0.91 \times 0.90}} = 0.96$. This indicates that $0.96^2 = 0.92$ or 92% of the variance of each method is due to the factor *common* to the two methods, and only 8% to factors *specific* to each method. More important is the correlation of the Starr ballistocardiograph stroke volume with the catheterization stroke volume (11). For 14 subjects, covering a wide age range, and including normal people and sick patients, the figure was 0.94. The figure is slightly inflated by the average of two ballistic records being taken, and by the inclusion of aortic cross-section in the old Starr formula. This figure, corrected for attenuation, reaches unity, and if not inflated from these causes, could scarcely be under 0.95. Lastly, the Nickerson low-frequency critically-damped ballistocardiograph gave a correlation coefficient of 0.83 with the catheterization figure for 54 observations over a wide age span of patients without heart disease. This corrected would again be of the order of 0.95, and at least with this instrument "variations in arterial pressure, heart rate and age appear to cause no significant variation in the relationship between the cardiac index as measured by the ballistic method and as determined by the catheter method" (8). Thus these methods agree well, at least for healthy persons at rest; and it is from these that we obtain normal standards and measure our degree of abnormality.

The fallacy of the present ratio standard

Two expressions have so far been proposed for cardiac output standards; the output per surface area (12) and the output per body weight (5). The implications of the use of these *ratios* as standards do not seem to have been generally realised: the subject has been discussed at length

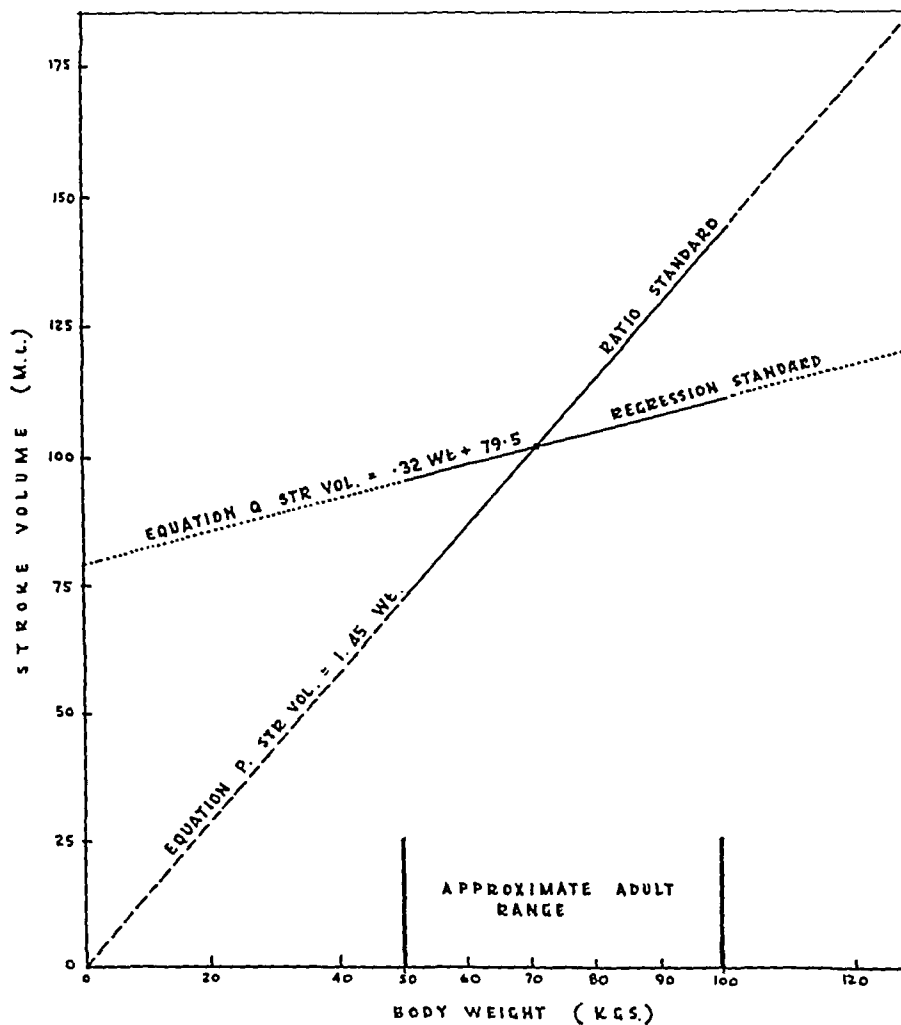


FIG. 1. THE RELATION OF STROKE VOLUME AND BODY WEIGHT

elsewhere (1). Except in a special case, not met by cardiac output figures, the ratio standard creates of itself a tendency to say large men have small stroke volumes and small men large ones and thus leads to the situation where large and small men are reported as abnormal more readily than are the medium sized.

This comes about in the following way. Figure 1 shows the stroke volume of the heart plotted against body weight (data of Table I). The use of the ratio, per-weight, standard implies that in the normal person, the stroke volume is proportional to the weight: in fact that the expression,

$$\text{Stroke Volume} = k. \text{Wt.}, \quad (P)$$

holds good over the range of values of weight for which the standard is used; that is, for all normal adult values. The constant k is determined by the mean values of the series of data on which the standard is founded. Thus the line in

equation P passes through the point of the two means, and, by virtue of the form of the equation, through the origin also. If this line were really a newly constructed per-weight standard, we should judge any given stroke volume as normal or abnormal according to how far away from the line our value fell. This is the current practice, using either the per-weight or per-surface area standard. Now actually this expression by no means represents the mathematically "best" or "true" relation between stroke volume and weight over the range of adult values. The best relation is that given by the regression equation (assuming rectilinearity of regression, which is justified at least as a first approximation), and this equation is:

$$\text{Stroke Volume (ml.)} = .32 \text{ wt. (kg.)} + 79.5. \quad (Q)$$

This is the line called equation Q in Figure 1, and it will be seen that it only coincides with that of

equation P at one point, the means. Equation P should be our proper standard, and it can be seen from the figure that a heavy man of 90 kg. will be given by the ratio standard a "normal" stroke volume which is too high, by as much, in this instance, as 20%. At the end of this paper the example chosen to illustrate the application of the new standards also illustrates this point.

The regression equation also has the advantage that a precise test of significance of the difference between the values found and those predicted is available. That is, confidence limits can be precisely specified, such that only one normal man in 20, or one in 100, as is wished, is reported wrongly as abnormal. A similar test is not so readily available for the ratio standard.

The multiple regression equation standard in theory

Correct normal standards of cardiac output should be constructed, then, from the regression equations found experimentally in healthy people. We not only wish to have standards for every weight, but also for each age, for either sex, and indeed for the varying conditions of all the variables upon which, in the normal individual, stroke volume depends. The more precise the standard for the normal, the smaller the variation we shall be able to identify as abnormal, and the earlier the diagnosis of disorder. What we need is a multiple regression equation in which is shown the dependence of stroke volume upon many variables, each with its own effect separated off from the effects of the others. Stroke volume, we may say, is given (in the normal) by

$$V_s = aL + bM + cN + dO + eP, \quad (A)$$

where L, M, N, etc. are such variables as weight, age and sex, and a, b, c, etc. are coefficients. Such an equation assumes that the relationships between all the variables concerned are linear, though there is no reason why more complicated functions either stated curvilinearly or reduced to linearity by taking logarithms or by some other means should not be used if they represent the data better. Our present data warrant no other assumption than that of linearity, except possibly in the case of age. Multiple regression equations of this sort are used in the newer tables of children's weights, and have

been constructed by pathologists to help them identify abnormal tissues (13). A very full and exemplary treatment of their construction and use for basal metabolism standards will be found in Harris and Benedict's monograph (14).

Cardiac output per minute is a physiologically important but composite variable, and its components, stroke volume (V_s) and heart rate, are governed by factors which are to a large degree independent of each other (as signified by V_s and heart rate having a negative correlation at rest of about -0.4). Both stroke volume and heart rate are of very considerable interest in themselves, and it would therefore seem best to have standards for these two variables, as well as for cardiac output per minute. It could then immediately be seen whether a person with a high cardiac output had it because of a high heart rate for, let us say, his age and sex, or a high stroke volume for his size, age and heart rate.

The variables that enter into the multiple regression equations depend, of course, on the correlations found empirically for healthy people. But *a priori* we may surmise that we should allow for age, sex, and body size and shape. Height, weight and surface area are merely crude ways of considering physique; these relations will be discussed in more detail in the next section. Our theoretical equations may then be:

$$\begin{aligned} \text{Stroke Volume} &= a. \text{ Body Size factor} + b. \text{ Age} + c. \text{ Heart rate} + d. \text{ Chest dysplasia} + e. \text{ Androgeny of Physique} + f \dots\dots\dots (B) \\ \text{Heart Rate} &= a'. \text{ Body Size factor} + b'. \text{ Age} + c' \dots\dots\dots (C) \\ \text{Cardiac output/min.} &= a''. \text{ Body Size factor} + b''. \text{ Age} + c'' \dots\dots\dots (D) \end{aligned}$$

It is proposed to derive equations of this type for each sex with the accuracy the data available permits.

The data

In Table I are listed the data for 50 healthy students. These determinations were made between 12 noon and 3.30 p.m. in June with laboratory temperatures varying from 21.2° C to 33.5° C. The subjects had taken no strenuous exercise on the day of the test, and had not smoked or had anything to eat or drink for three hours previously. Each sat in a chair resting for 30 minutes and then lay down on the ballistocardiograph. At the end of 14 minutes a recording was taken, and another at the end of 21 minutes. The figures in Table I are the average of these two determinations (3).

TABLE II
Cardiac output data on healthy men and women

Authors	Starr	Tanner	Starr	Lewis	Grollman	Starr	Stead	Courmand
Method	Ballistocardiograph			Acetylene		Ethyl iodide	Catheterization	
Sex	Male	Male	Female	Male	Male	Male	Male	Male
Number of Subjects	108	50	66	80	37	22	10	13
Age (years)	40.4 Mean 12.7 Range 20-79	22.8 1.5 20-27	35.0 11.6 21-68	59.0 11.1 40-79	ca. 25 ca. 3.0	31.3 8.6 21-51	25.0 4.4 22-35	38.1 8.7 21-52
Surface Area (sq.m.)	1.89 MI .13	1.85 .12	1.61 .14	1.79 .14	1.81 .13	1.83 .13	1.93 .09	1.76 .17
Heart Rate (per min.)	70.8 MI 9.0	61.7 8.2	71.8 9.1	60.3 7.0	62.0 6.6	63.9 8.3	70.3 7.6	66.0 7.2
Stroke Vol. (V.) (ml.)	81.5 MI 15.5	101.8 9.8	68.2 8.4	68.4 10.4	65.2 7.7	71.5 21.3	85.6 11.7	84.0 16.1
Cardiac Output (C.O.) (l/min.)	5.73 MI 1.10	6.21 .79	4.88 .83	4.07 .51	4.01 .35	4.47 1.05	6.00 .99	5.51 1.09

First order regressions, stroke volume																
V _s on S. Area	25	11.3	26	11.2	13	7.3	28	7.8	11	9.8	41	35.4	23	45.0	56	23.3
V _s on Heart Rate	-.64	.16	-.49	.16	-.12	.11	-.86	.14	-.81	.14	-1.5	.46	-.29	.53	-.58	.65
V _s on Age	-.82	.090			-.35	.080	-.10	.11			.47	.54			-.89	.49

Partial regressions, stroke volume																
V _s on Surface Area (HR, age const.)	21	7.9	28*	10.2	24	6.2	29	7.1	21*	8.3	56	28.8	23*	47.5	40	20.6
V _s on Heart Rate (SA, age const.)	-.51	.11	-.45*	.15	-.21	.093	-.96	.12	-.91*	.16	-1.5	.45	-.29*	.56	-1.0	.51
V _s on age (SA, HR const.)	-.77	.082			-.43	.076	-.06	.077			.27	.46			-.74	.43

Table II sets out the biometric relations of all available studies which give full figures, including the heights and weights of the subjects. The first and third columns refer to the data of Starr, the second to the contents of Table I. These three columns are results from Starr's high-frequency ballistocardiograph, using the area formula of Starr, omitting A, the aortic cross-section area, and using 100 instead of 33 as the constant, the reason for which is given below.

The fourth and fifth columns refer to acetylene method results. Lewis studied 100 apparently healthy middle-aged and old men (15), but only the data on the 80 subjects between the ages of 40 and 79 figure in the Table II calculations. Grollman's subjects were mostly medical students in the post-absorptive state (12). The sixth column lists data obtained by Starr and his colleagues using ethyl iodide (16), and the last two columns are from cardiac catheterization studies.

Very few catheterization data are available on normal subjects. This being the case, it is all the more unfortunate that the two available series, due to Cournand *et al.* (17) and Stead *et al.* (18), do not agree. Stead's value for stroke volume for ten normal subjects ([18] their group 1 plus WP of group 4) of mean surface area 1.93 sq.m., heart rate 70.3 and age 25, was 85.6 ml. Cournand's 13 subjects' mean stroke volume, when adjusted to Stead's means of surface area, heart rate and age, is 96.2 ml. or 96.6 ml. according to whether the adjustment is made using the regressions of Cournand's sample itself or of equation B3, below. In either case the difference between Stead's and Cournand's means is more than twice its standard error, and unlikely to be due to chance; it stems from a difference in arterio-venous oxygen difference, due to method, climate, choice of subjects, or some other factor. Thus the two series cannot be considered as from the same population and must be dealt with separately.

In the upper part of Table II means and standard deviations of the various variables are tabulated; the standard deviations are figures for the sample. The heart rates serve as a guide to the degree of rest and relaxation of the subjects; in three of the four large male series they are satisfactory, but Starr's ballistocardiograph group have rather high rates, perhaps because they were not in the post-absorptive state.

The middle part of Table II lists the regressions and partial regressions that we are concerned with, and which constitute the most important part of our analysis. These regressions are estimates of regressions in the population from which each sample was drawn, but some are, of course, much better estimates than others, due to the relative size of the samples. The standard errors are the figures in italics each below and to the right of the parent regression coefficient. The sampling error of the catheterization data in particular is distressingly large in relation to the making of standards.

The lower part of Table II lists various correlation coefficients of interest, and provides a way of judging the goodness of fit of the regression lines specified. The level of r for which $P = .05$ is given, and for coefficients

of this size the figure for twice the standard error of each coefficient will not be very much smaller than this.

In Table III are the more detailed statistics for age changes in cardiac output from the studies of Starr and of Lewis.

BALLISTOCARDIOGRAPH STANDARDS

Leaving a further consideration of Table II to the last part of the paper, we will now develop the appropriate standards for the Starr ballistocardiogram.

The revised ballistocardiogram formula

The dropping of A, the cross-sectional area of the aorta, from Starr's ballistocardiogram formula (19) leaves the value of the constant in the formula indeterminate, and before we can give regression coefficients for stroke volume we must adopt some particular figure. The value to be chosen is that which will bring the mean normal figure for stroke volume calculated from the ballistocardiogram into line with the mean obtained by the catheter technique.

Using the Starr area formula without aortic cross-section and omitting the constant 33 (*i.e.*, $V_s = \sqrt{(2I \text{ area} + J \text{ area})} \sqrt{c}$ where the areas are measured in millimeter-seconds, and 280 gm. displace the light spot 1 cm.), the mean value for Tanner's 50 men of average age 23 is 1.02 ml., and for Starr's 24 men (Table III) of average age 24 is .92 ml. When both these means are adjusted for surface area and heart rate differences by equation B3, so that they correspond to the value of a group of surface area 1.80 sq.m. and heart rate 62, Tanner's series mean is 1.00 ml. and Starr's .94 ml. The difference between the two series is just twice its standard error; probably Tanner's subjects have higher outputs because they were all measured in the summer. A figure of about .96 ml. would perhaps be the best all-the-year-round estimate. The question is whether to bring this up to Stead's mean catheter figure, or to Cournand's. I have chosen, pending more data, to bring it to the latter, chiefly because this makes the constant the convenient and self-evidently approximate figure of 100. It is recommended that this be used in Starr's area formula, until further developments take place.

TABLE III
Changes in cardiac output with age

		Starr: Ballistocardiograph							Lewis: Acetylene					
		Males					Females							
Age		20-29	30-39	40-49	50-59	60-79	20-29	30-49	50-69	40-49	50-59	60-69	70-79	80-89
Number		24	29	29	18	8	29	27	10	20	20	20	20	20
Surface Area	M	1.86	1.91	1.92	1.91	1.85	1.57	1.61	1.72	1.82	1.82	1.79	1.75	1.68
	σ	.13	.11	.14	.12		.14	.12		.14	.13	.15	.13	.15
Heart Rate	M	70.6	71.5	67.8	72.2	77.5	73.6	70.7	69.5	61.1	60.8	60.1	58.7	62.9
	σ	7.3	9.6	7.9	9.0		10.0	8.1		5.1	8.1	7.5	6.8	9.0
Stroke Volume	M	92.4	86.7	82.1	66.8	48.5	70.4	67.4	62.8	71.3	67.4	68.1	66.5	60.0
	σ	10.5	10.8	13.6	12.6		7.8	8.5		10.2	10.9	9.0	10.5	10.3
Cardiac Output	M	6.51	6.19	5.52	4.75	3.74	5.19	4.76	4.34	4.32	4.04	4.06	3.86	3.74
	σ	.88	.97	.86	.69		.85	.77		.57	.37	.48	.47	.65
I wave area	M	21.7	19.9	17.0	9.7	9.4	12.2	11.3	8.4					
	σ	6.9	5.4	7.5	4.4		2.9	3.0						
J wave area	M	50.1	42.4	39.6	30.0	24.6	32.2	27.9	25.9					
	σ	10.2	12.4	9.2	8.8		7.8	5.8						
I/J		2.3	2.1	2.3	3.1	2.6	2.6	2.5	3.1					
Average blood pressure	M	93.8	96.9	94.4	97.4	111.4	91.2	96.0	106.5	91.1	97.6	94.8	105.6	114.0
	σ	7.8	5.7	8.4	7.3		6.9	9.7		6.6	7.9	8.5	11.2	13.0

This new constant is probably too high; it assumes that persons lying at rest with an intra-cardiac catheter in place and a needle in the femoral artery are as truly basal as those lying in perfect comfort on the ballistocardiograph, and this is unlikely to be true. It places the mean level for ballistic stroke volumes considerably above that obtained by adding 18.5% to the old Starr formula, as suggested by Cournand (11), which gives a value of 60 for the constant, and leads to the rather low figure of 3.7 l/min. for the cardiac output of healthy young men at rest. (Current model experiments, Professor Starr tells me, support the figure of 60, all the same.) That at present we have no means of knowing absolute values from the ballistocardiogram is of no importance whatever so far as our standards are concerned. As used in practice they are independent of the constant and of any absolute level. When the constant changes as a result of further data accumulating, the regression coefficients in equations B3 and C3 below will change proportionately, but the standard error of estimate (see below, p. 577) will do likewise and the probability of a patient being abnormal will remain unaltered.

The relation with body size

There are two classifications of physique which command attention at the present time. One of these is due to Sheldon (4), and the other, resulting from applying the methods of factor analysis to measurement of the body, has reached its most advanced position in the hands of Burt (20). A discussion of the principles of these systems and of the relation between the two will be found elsewhere (21). The factor analysis yields a general Body Size factor, and several subsidiary factors such as the leptosomic, or linearity factor, which is the equivalent of Sheldon's ectomorphy. The Size factor is a measure of the simple largeness of the body; every measurement taken on the body correlates positively with it. It is really this Size Factor that we are attempting to consider when we regulate the dosage of a drug by body weight or calculate cardiac output per kilogram or per square meter. (We may thereafter, it is true, proceed to consider the relative amounts of more or less active tissue, the bone, muscle and fat proportions, but only *after* the general size has first been allowed for, or, in statistical language,

partialled out.) An estimate of a man's Size Factor score may be obtained from a regression equation involving height and weight and sacrum-heel length (22) and it is this Size Factor estimate which we should use as the first term in our multiple regression equation for cardiac output. However, another expression has been substituted, and this the familiar one, DuBois Surface Area (23). This surface area expression can be shown for men to correlate with the Size Factor estimate to the extent of .98 and its use therefore gives precisely the same result: it is a trifle simpler to measure and compute and is already familiar to clinicians. It should be again emphasized, however, that the surface area is used simply because it provides a good estimate of the Size Factor and for no other reason. There is no implication whatever of theoretical physiological relations concerning surface area and cardiac output, such as caused controversy in the field of basal metabolism (14, 23, 24). The figure used for weight should be the subject's usual weight in health: under no circumstances should any so-called "ideal" weights be used, which are merely methods of telling what weight a given person would have if he were somebody else of entirely different appearance. I have used surface area as equivalent to the Size Factor for women as well as men, and the propriety of doing so might well be questioned: data on the subject are lacking.

The regression of stroke volume on surface area for ballistocardiograph data can be seen in Table II. The figure for women is less than that for men, but this seems to be due to the existence of a positive relation between surface area and age in the series of women, but not in the men. Partial regressions with age constant give almost identical figures for both sexes.

Heart rate and body size seem to be independent of each other. The heart rate has insignificantly small correlations with height, weight and surface area in all the data of Table II. The large series of Boas and Goldschmidt (25) and Harris and Benedict (14) gave almost exactly zero correlations. We may conclude that resting heart rate in the human is unrelated to the Size Factor.

Beyond the general Size Factor, one may suspect that a person's *body build* may influence his cardiac output. In the 50 young men of my series, however, no sure relation could be demon-

strated between heart rate, stroke volume or cardiac output and any of the Sheldon components (3). Correlations with Burt's leptosomic factor scores (taken to increase as linearity or ectomorphy increases, contrary to the usage in my previous study [21]) were only .18 for stroke volume and .19 for cardiac output, either of which would be expected to occur once in five samplings of a population in which there was no correlation at all; and .06 for heart rate. Thus at present, though some quite small positive relation between stroke volume and linearity of build may exist, the inclusion of body build factors other than general size would not substantially improve our standards.

Stroke volume and heart rate

The relationship between stroke volume and heart rate is an inverse one. There is good agreement, particularly in the partial regression, between the two male ballistocardiograph series, but the figure for women seems only to be about half as large.

Stroke volume and age

The regressions and correlations are in Table II and some more detailed statistics in Table III. Starr's male data show a very marked decrease with age, with a linear correlation of $-.65$. The female regression is again about half the male.

The standard regression equations

We are now in a position to assign values to the coefficients in the regression equations B and D, for the ballistocardiograph. These values are the partial regressions in Table II; but they have been rounded off to avoid giving an unwarranted appearance of high accuracy. The figures for stroke volume on surface area are sufficiently close for the value 25 to be used for males and females; for stroke volume on heart rate, $-.50$ covers adequately both male series. We then have:³

$$\text{Estimate } V_s \text{ (ml.)} = 25 \text{ (S. Area} - \text{Mean S. Area)} - .50 \text{ (H. Rate} - \text{Mean H. Rate)} - .80 \text{ (Age} - \text{Mean Age)} + \text{Mean } V_s \text{Men, ballisto (B1)}$$

³ A set of somewhat different equations based on preliminary calculations has appeared in the Proc. Phila. Physiol. Society, Amer. J. M. Sc., 1948, 216, 598. The present equations replace those preliminary ones.

and

Estimate V_s (ml.) = 25 (S. Area - Mean S. Area) - .20 (H. Rate - mean H. Rate) - .40 (Age - Mean Age) + Mean V_s Women, ballisto (B2)

The partial-regression coefficients in the equations refer to population values, but the figures to be inserted for the means of stroke volume, surface area, heart rate and age should perhaps be taken by each separate laboratory from the usual run of its own data. In this way, though the mean stroke volume figures may differ in different laboratories due to variations in method, climate and so forth, estimates of V_s will always be couched at the same level as the actually recorded measurements. We must perforce assume that the standard deviation is unaffected by such changes in the mean as we have to deal with. For heart rate, at least, the figures of different series agree very well, both as to mean and standard deviation (3, 12, 14, 25). The population mean for resting males may be taken as approximately 62, and the standard deviation as about 8. For women these figures are 69 and 9. These values only apply if really sufficient time of resting is allowed the subject before determination; at least 15 and perhaps 20 minutes. The subject should preferably be in the post-absorptive state.

Using 100 as the constant in the ballistocardiograph equation, the mean of Starr's series is 82 ml. for men and 68 ml. for women. Putting in the means of his samples also for surface area, heart rate and age, we finally have:

Estimate V_s (ml.) = 25 S. Area - .50 H. Rate - .80 Age + 103 Men, ballisto (B3)
Estimate V_s (ml.) = 25 S. Area - .20 H. Rate - .40 Age + 56 Women, ballisto (B4)

These equations, as has been emphasized, should be regarded only as major terms in longer, more accurate, expressions. They are inapplicable below the age of 20, and of doubtful validity above the age of 70. The standard errors of the coefficients have been indicated in Table II. The multiple correlation of V_s in these equations is .77 for men, and .50 for women; that is, surface area, heart rate and age account for 53% of the variance of stroke volume estimated by the ballistocardiograph in men, and 25% of the variance in women.

By substituting an individual's measurements in the relevant equation, we obtain an estimate

of what his stroke volume should be on the basis of his surface area, heart rate and age. We then discover experimentally his actual stroke volume. Naturally the actual stroke volume differs from the estimate by a certain amount, which we will call d . The question to which the clinician requires the answer, and to which all the above development has been leading is simply "What is the likelihood that this man's difference, d , is due merely to sampling from the normal population?". This is a more valid form of the cruder question "Is this man's stroke volume normal or not?", for if the likelihood of the man's difference having arisen just by sampling is small, the man is presumably *not* from the normal population, and is thus from another, abnormal one. It has been customary, with the ratio standard, to say that the man is abnormal if the difference between his actual value and the mean value, or standard, is 20% of the mean. This usage has little statistical foundation, and with the regression equation standard we can replace it with a more valid and more informative device. The estimates we make from the regression equation have a standard error, and a guide to the likelihood of a given man *not* coming from the normal population is directly given by the ratio of the difference, d , to this standard error. The standard error is given by

$$\sigma_{est} = \sigma_{V_s} \sqrt{1 - R^2}, \quad (L)$$

where R is the multiple correlation coefficient. Using the figures already given, we have $\sigma_{est} = 16\sqrt{1 - .53} = 11$ ml. for men and $8.6\sqrt{1 - .25} = 7.5$ ml. for women.

The interpretation of d/σ_{est} is that of the ordinary critical ratio and from it the clinician can tell how much confidence to place in the idea that the man may be abnormal. The confidence limits usually used by statisticians are ± 2 for the ratio, which would lead to five normal men in 100 being classed as abnormal. Thus we may roughly place the limits of stroke volume normality for males at ± 22 ml. from the regression estimate. (Though these limits seem reasonably appropriate, they are quite arbitrary and stricter ones may be taken if the clinician desires: for example at ± 2.5 , which would lead to wrong classification only once in 100 people.)

Heart rate. To complete equation (C) for predicting heart rate, we need only to employ the

mean figures for both sexes. The standard deviations of the distributions of heart rates in the population are known. Again, each laboratory should use its own mean values. Whether or not a given heart rate should be considered abnormal may be tested in the same way as for stroke volume.

Cardiac output. The equivalent equations for cardiac output are:

$$\begin{aligned} \text{Estimate C.O. (1/min.)} &= 1.1 (\text{S. Area} - \text{Mean S. Area}) \\ &- .05 (\text{Age} - \text{Mean Age}) + \text{Mean C.O.} \dots\dots\dots \\ &\dots\dots\dots \text{Men, ballisto (D1)} \end{aligned}$$

and

$$\begin{aligned} \text{Estimate C.O. (1/min.)} &= 1.7 (\text{S. Area} - \text{Mean S. Area}) \\ &- .04 (\text{Age} - \text{Mean Age}) + \text{Mean C.O.} \dots\dots\dots \\ &\dots\dots\dots \text{Women, ballisto (D2)} \end{aligned}$$

With the mean values of Starr's series inserted, these become:

$$\begin{aligned} \text{Estimate C.O. (1/min.)} &= 1.1 \text{ S. Area} - 0.5 \text{ Age} + 5.5\dots \\ &\dots\dots\dots \text{Men, ballisto (D3)} \end{aligned}$$

$$\begin{aligned} \text{Estimate C.O. (1/min.)} &= 1.7 \text{ S. Area} - .04 \text{ Age} + 3.5\dots \\ &\dots\dots\dots \text{Women, ballisto (D4)} \end{aligned}$$

These equations like those for stroke volume are subject to later modification of coefficients as more data accumulate, and are applicable only between the ages of 20 and 70. The multiple correlation coefficient for men is .61 and for women .54, surface area and age thus accounting for 37% of the variance of cardiac output in resting men, and 29% of the variance in women. It may be recalled that a similar equation for basal oxygen consumption yields a multiple correlation of about .85 which accounts for 73% of variance (14). Thus our standards are only about half as effective at catching the abnormals as are the B.M.R. standards. Any future cardiac output standard that does detect abnormals as efficiently as do the present B.M.R. standards will have to be very much more complicated than those developed here.

The last point may be brought home by a consideration of the interpretation of d , the difference, as before, of the actual cardiac output from the estimated. Again we have

$$\sigma_{\text{est}} = \sigma_{\text{C.O.}} \sqrt{1 - R^2}. \quad (\text{M})$$

The higher R , the smaller σ_{est} , and thus the smaller the difference, d , to place a man outside the normal limits. We have this time $\sigma_{\text{est}} = 1.1 \sqrt{1 - .37} = .87$ l. min. for men and $.84 \sqrt{1 - .29} = .71$ l. min. for women. Confidence limits would

again be ± 2 for the ratio d/σ_{est} . Thus the limits of cardiac output normality for males would be roughly at a ± 1.7 l/min. difference from the regression estimate for men, and 1.4 l/min. difference for women.

Use of the standard equations. We may conclude this section with a couple of practical examples of the use of the new standards. Subject No. 5 of the Tanner ballistocardiograph series was aged 23; his height was 176 cm., weight 77.3 kg., and Dubois surface area 1.92 sq.m. After 20 minutes lying at rest, his heart rate was 73 per min. and calculation from his ballistocardiogram impacts gave a stroke volume of 91 ml.

To answer the question whether this man is abnormal substitute his surface area, heart rate and age in equation B3 using the Starr series means. The estimated stroke volume figure for such an individual is 96 ml. The difference between the value found and that predicted is 5, and the critical ratio of this difference to its standard error is $5/11 = 0.5$. Thus there is no reason to regard this man as abnormal. The report on this man would read "actual stroke volume 91 ml., standard estimate 96 ml., critical ratio 0.5, normal." It is instructive to consider how this man would have fared by reference to the old standards. His per-surface area estimate is 106 ml. and his per-weight estimate no less than 112 ml. On the basis of the weight standard he would be judged abnormal, with a stroke volume 21% below average.

A second man, F., aged 69, of surface area 1.89 sq.m., had a heart rate of 64 and a stroke volume estimated by the ballistocardiogram of 42 ml. He complained of nothing, worked normally, and was tested merely to add to the collection of normal data. His stroke volume, however, seems small. Is it likely he is abnormal? Substitution in equation B3 gives his regression estimate as 63 ml. The difference between the values found and estimated is 21 ml. and the critical ratio is $21/11 = 1.9$, on the borders of conventional statistical significance.¹ It is therefore relatively un-

¹ This is using the hypothesis that the man is *abnormal*, i.e., has either too low or too high a stroke volume. But if the hypothesis is that his output is too *small* (probably the more important one clinically, since other factors will usually lead one to suppose abnormality of one particular sort), then the level of normality should be taken at

likely that this man has a normal heart, despite his history; and, indeed, a few years later, he became aware of having coronary disease.

The cardiac output estimates for these two men may be calculated from equation (D3) and the likelihood of their being abnormal similarly assessed; the information yielded for these two cases is the same as above.

COMPARISON OF BALLISTOCARDIOGRAPH WITH OTHER METHODS

A few of the relations between the various cardiac output methods implied by the sea of figures in Table II seem worth fishing up and examining shortly, even if their subsequent fate is to be thrown back in. Most of this discussion concerns males only.

Stroke volume and surface area

On the present data there is little reason to suppose that any significant difference in this regression exists between the ballistocardiograph, acetylene and catheterization methods. Cournaud's series had a curiously large range of surface areas, with a sample standard deviation of .17 compared to all the other samples' .13 or .14. Perhaps this was done on purpose with the idea of covering a good range of sizes in normals; it may well be responsible for tipping up the regression line if the relationship is not really quite as linear as we would like to suppose.

There is a suspicion that the ballistocardiogram area formula may lead to higher correlations with height and lower with weight than do the other methods; possibly the length of the body influences the form of the I or J waves. Starr has himself suggested that body length should be allowed for in the ballistocardiograph formula (19), and Nickerson has included it in a revised formula for use with his low frequency instrument (8). A long aorta contains a greater mass of blood than a short one, and it is conceivable also that the time between the blood hitting the arch and the bifurcation should be larger in tall men than in short ones. Calculations on my series of 50 subjects does indeed show that the J wave area has with bodily stature a specific relation of its own: a relation, that is, not shared by the I wave.

a critical ratio of 1.65 instead of 2. One then misclassifies as too low outputs of five people in 100.

Stroke volume and heart rate

There appears to be a significant difference in this regression between the acetylene and the ballistocardiograph methods. As heart rate rises, stroke volume apparently decreases nearly twice as fast by the acetylene as by the ballistocardiograph. At least part of the reason for this seems clear enough. In both acetylene studies the second samples of rebreathed air were taken 23–25 seconds after the beginning of rebreathing, and the evidence presented by Hamilton (26) that considerable recirculation has occurred during this time seems entirely convincing. The greater the heart rate, the less circulation time (27–29), the more the recirculation, and therefore the lower, apparently, the stroke volumes: part of the negative correlation in the acetylene data is artefactual. For this reason I would be inclined to place the most likely partial regression for the catheter method near to $-.5$ rather than -1.0 ; the differences in the two catheter series are presumably due mainly to sampling error.

Stroke volume and age

There are only two considerable sets of data on age changes, and unfortunately they are in complete disagreement. Starr's ballistocardiograph data show a large decrement with age, while Lewis's acetylene data have hardly any decrement at all (Table III).

The disagreement in results is probably due to both sampling bias and to the difference in method. Lewis's subjects on which the regressions are calculated covered the age range 40–79 and were selected as being healthy men free from cardiovascular as well as other symptoms. It is perhaps not unreasonable to suppose that towards the end of the age span the subjects so qualified were those who had maintained fairly high cardiac outputs. Others, who would enter into lower age group standards as perfectly normal, and who subsequently had suffered a (not unusual) decline of output might perhaps not be free of slight symptoms. In short, there may have been selection for high cardiac outputs at the upper end of the age distribution. Secondly, in the acetylene method the amount of recirculated blood may well decrease with advancing age (27, 28, 30) and if so cardiac outputs would appear to increase.

Thus a true drop would be marked by an artefactual rise.

The only catheterization figure, from Cournaud's series of 13 subjects between the ages of 21 and 52, agrees with the ballistocardiogram figure, but its standard error is so high that the agreement cannot be taken very seriously. Somewhat in agreement's favour, however, are the data from the low-frequency ballistocardiograph of Nickerson (8). The correlation of these results and catheterization was .83 (which corrected for attenuation reaches .9) and no change occurred with increasing age in the relation between the results secured by the two methods.

There is on the other hand an argument in favour of the ballistocardiograph overemphasizing the decrement with age, and doing so in a way which may prove of great value to clinicians in the future. The area under the I and J waves reflects not only the stroke volume, but also the ejection curves of the ventricles (31). If the ejection curve changes, the formula for stroke volume becomes relatively inapplicable. Though as yet there does not seem to be any direct evidence that the ejection curve *does* change with age, that it should do so would be entirely consonant with general ideas of aging, and indirect evidence may be found, perhaps, in electrocardiographic changes (32). Such a change must be of the sort which will lead to a diminution of the area under the I and J curves, even when the actual stroke volume remains constant. It must not, however, alter the ratio of the J area to the I area, since this remains more or less unchanged as age advances (Table III). To satisfy these requirements the nature of the change can be predicted on the basis of Hamilton's curves (31, Figure 8) to consist of a ventricular contraction of usual initial speed and force but less sustained than normal; and this seems not an unreasonable thing to occur with myocardial aging.

Probably the true partial regression of stroke volume on age lies between .6 and .8 ml. per year, over the age range considered. This assumes a linear decrease of stroke volume with age, which, though unlikely to be actually true, is the only assumption warranted by the extant data. It also implies the conclusion that the arteriovenous oxygen difference rises with age,

since the oxygen consumption correlation is only about $-.3$ (33) compared to the stroke volume figure of between $-.5$ and $-.65$. Pending further developments, it would be best to use the same regression for cardiac catheter method standards, if these are needed, as for the ballistocardiogram, remembering this may exaggerate the age effect slightly. There is great need for simultaneous studies by the ballistocardiograph and catheterization methods upon normal subjects of different ages. When the position is clarified, it may well be that one of the chief clinical values of the ballistocardiogram will lie in measuring not stroke volume, but myocardial changes and efficiency.

Cardiac output and sex

The only extensive data on the cardiac output of normal women are those of Starr. When the stroke volume of the 20–29 year old women is adjusted by means of equation B4 to the same surface area and heart rate as Starr's 20–29 year old males, it is smaller than the male figure in the ratio .85. The corresponding ratio for the 30–49 year old women is .89.

The resting heart rate of women, on the other hand, is about 10% higher than that of men (14, 25), and since heart rate is not related to body size, this is irrespective of size. Thus the cardiac output per minute would be more nearly the same than is stroke volume. The ratios for Starr's groups are smaller than would be expected, probably because the subjects have greater than basal heart rates; they are .87 and .91 for the two age groups.

Heart rate and stroke volume seem to be more independent in women than in men, but this result should be treated with reserve, as the difference between the two regressions lies only at the level of $P = .075$ even if the comparison is made with both Starr and Tanner male data. Stroke volume in women apparently declines somewhat less rapidly with age than in men, and in this case the probability of the regression difference having arisen by chance sampling is only 1 in 70 ($P = .014$). The equivalent partial regressions of cardiac output on age, however, are not significantly different.

Standards for catheterization data

The data are too meagre and in too little agreement to warrant the creation at present of special catheter standards. It is recommended that the ballistocardiograph standards be used with catheter data pending further developments, and with full realization of the possible errors incurred. The fact that in some disorders the ballistocardiograph may give results widely different from the catheter, due to changes in the cardiac ejection curve, is irrelevant so far as the use of normal standards is concerned: under such circumstances the ballistocardiograph standards would relate better to the catheter results than to the ballistic ones. With the latter, one can imagine a situation where a true increase in stroke volume may be accompanied by a weakened ejection, one fault compensating for the other. Ultimately standards for the ejection curves themselves will have to be made.

SUMMARY

1. Ballistocardiographic cardiac output data, blood pressure, mouth temperature and somatotype are reported for 50 healthy young men at rest.

2. The reliability of cardiac output methods is discussed, and it is shown that agreement between the ballistocardiograph and catheterization methods is very good, if their respective reliabilities are taken account of.

3. The present use of ratio standards such as per-weight and per-surface area involves a fallacy and leads to erroneous results, sometimes of practical importance; this is discussed.

4. The construction of normal standards for cardiac output by means of multiple regression equations is discussed and the appropriate standards derived for the Starr ballistocardiograph stroke volume and cardiac output for men and women. Their clinical use is illustrated.

5. To bring the Starr ballistocardiograph data into line with Cournand's catheterization values, it is recommended that, when aortic cross-section is dropped from the Starr wave area formula for stroke volume, the constant 33 be provisionally replaced by 100.

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AN EVALUATION OF HUMAN SERUM ALBUMIN IN THE TREATMENT OF CIRRHOSIS OF THE LIVER^{1,2}

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INTRODUCTION

The recent development of normal human serum albumin for intravenous administration has led to its use in treating clinical states marked by hypoalbuminemia, including chronic liver disease (1-4). Previous work with this therapeutic agent has been confined to relatively short-term studies. The present study was undertaken to determine the effect of salt-poor human serum albumin on ascites and edema in chronic liver disease when administered in sufficient amounts to raise the serum albumin concentration and maintain it at or near normal over long periods of time. The results of such therapy attempted in 29 patients with cirrhosis of the liver are presented in this communication.

METHODS

Patients were selected who had marked evidence of hepatic cirrhosis by history, physical examination, and liver function tests, and who had ascites, edema, and hypoalbuminemia. In a few patients the diagnosis was confirmed by liver biopsy or at post-mortem examination. In most instances patients were chosen in whom there was no response to other forms of therapy. In the majority of cases, the need for repeated paracenteses was established before treatment was initiated. During the period of study no other therapeutic agents were given

except for a nutritious diet which the patients were urged to eat. A few patients were given mercurial diuretics at infrequent intervals but with only a temporary effect on the course of their fluid retention. The salt-poor concentrated (25 per cent) human serum albumin was given intravenously, diluted with an equal volume of 5 or 10 per cent dextrose solution. Quantities of albumin necessary to raise the serum albumin concentration to between 3 and 4 grams per 100 cc. varied: usually from 50 to 100 grams daily were given over a five to ten day period. The majority of patients received 75 grams per injection and thereafter 75 grams once, 50 grams twice or 25 grams three times weekly. If marked improvement in ascites was not observed during the first ten days of treatment, maintenance injections were continued for from three weeks to eight months. In three patients the effect of a low sodium diet was studied during albumin therapy.

Abdominal paracentesis was performed as necessary and whenever possible the protein concentration was determined on the fluid obtained. The serum protein concentration and albumin and globulin fractions were determined at weekly intervals; total protein by micro-Kjeldahl analysis and albumin and globulin separation by Howe precipitation (5). Serum thymol turbidity (6) and serum bilirubin (7) were determined at weekly intervals. The percentage of bromsulphalein retention, 45 minutes following a 5 mgm. per kilogram dose, was determined at less frequent intervals. Most of the patients were weighed daily and frequent abdominal girth measurements were made. The latter were found to correlate well with weight gain and ascites formation.

RESULTS

Twenty-nine patients received albumin in varying amounts during periods of from four days' to eight months' duration. Table I shows the statistical results of the series. Twenty-five patients had both edema and ascites before therapy was begun and the remaining four had ascites alone. A history of heavy intake of alcoholic beverages was obtained in 25 cases, two admitted only a moderate alcoholic intake, while two denied drinking. In nine cases albumin administration had to be stopped because of untoward reactions, lack of cooperation, or untimely demise. Therefore, of

¹ The serum albumin used in this study was processed by the American National Red Cross from blood which it collected from voluntary donors. This is one of a series of investigations on serum albumin being carried out with material supplied by the American National Red Cross. As soon as sufficient data become available to justify final conclusions concerning its therapeutic value a full report to the medical profession on the use of serum albumin in medical practice will be published.

² This study was aided in part by a gift to Harvard University from the Abbott Laboratories, North Chicago, Illinois.

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TABLE I
Results of albumin administration to patients with cirrhosis of the liver

Patient	Total albumin given grams	Period of therapy	Paracenteses necessary*			Period of follow-up since therapy and end result
			Before therapy	During therapy	After therapy	
Immediate improvement						
S. P.	450	6 days	0	0	0	Free of ascites—11 months.
F. F.	375	5 days	0	0	0	Free of ascites—5 months.
S. H.	300	6 days	0	0	0	Died of bleeding varices 1 week after treatment.
J. G.	400	4 days	0	0	0	Recurrence of edema and ascites after 6 weeks. Died in coma 4 months later.
Slow improvement						
G. D.	1000	3 months	2(16)	0	0	Free of ascites—16 months.
P. B.	675	5 weeks	1	0	0	Free of ascites—10 months.
J. M.	1075	7 weeks	3(17)	1	0	Moderate ascites not requiring tap—8 months.†
A. D.	1500	6 weeks	1	0	0	Recurrence of edema after 6 weeks. Free of ascites—11 months.
H. H.	600	8 days	3(6)	0	—	Recurrence of edema and ascites after 6 weeks. Refractory to retreatment. Died of bleeding varices during therapy.†
Delayed improvement						
J. S.	1975	6 months	6(15)	4(24)	0	Free of ascites—6 months.
W. M.	3675	7½ months	4(23)	6(29)	0	Free of ascites—8 months.†
Improvement only with low sodium diet						
E. G.	2405	6 months	2(28)	3(33)	1	Died in coma 2 months later.
V. L.	1825	2 months	2(14)	1	—	Died of bleeding varices during therapy.
C. M.	4000	7 months	2(15)	9(28)	—	Still reforming ascites—under treatment.
Failure						
M. C.	1750	3 months	2(30)	7(15)	4(19)	Ascites increased with therapy. Died in coma 3 months later.
F. G.	350	5 days	3(30)	0	0	No apparent early benefit. Died in coma 2½ months later.
H. G.	900	3 weeks	4(34)	1	—	No apparent benefit. Died of bleeding varices 6 weeks later.
N. C.	900	3 weeks	3(16)	2(7)	—	Died in coma during therapy.
R. A.	525	18 days	1	1	—	Died of epistaxis 48 hours after last albumin.
P. W.	2300	4½ months	1	6(20)	—	Died in coma during therapy.
Indeterminate						
C. F.	425	12 days	3(33)	1	—	Pyrogenic reaction—coma. Died in coma 8 days later.
J. C.	450	7 days	1	0	—	Died during treatment from diphtheria infection of abdominal skin.
H. K.	450	9 days	2(15)	0	—	Pleural fluid increased with therapy. Left against advice—no follow-up.
R. V.	400	26 days	0	0	0	Intestinal obstruction during therapy. Died of int. obs. 2 months later.
B. B.	375	5 days	0	0	0	Improvement possibly spontaneous. Free of ascites—9 months.
L. B.	150	2 days	4(30)	0	0	Pulmonary edema with therapy. Spontaneous diuresis. Free of ascites—7 months.
J. Q.	225	6 days	0	0	0	Diuresis before therapy begun. Died in coma 2 weeks later.
P. M.	150	2 days	0	0	—	Pulmonary edema with therapy. Died in cardiac failure 1 day later.
F. H.	300	12 days	0	0	0	Improvement possibly spontaneous. Free of ascites—2 months.

* Average interval (days) between paracenteses given in parenthesis.

† Sodium balance studies were done on these patients who received a low sodium diet for short periods of time.

the total number, 20 patients are considered to have had sufficient therapy and follow-up to determine the effect of treatment. Improvement was judged on the basis of diminution in ascites and edema and other signs of improvement clinically and by laboratory tests. There was often a discrepancy between the decrease in fluid retention and improvement in other manifestations of liver disease. In this study the change in ascites and edema was used as the prime criterion of improvement.

The results presented in Table I can be divided into four main types: (1) immediate improvement with diuresis seen within the first ten days of treatment; (2) slow improvement with fluid retention disappearing (diuresis taking place) during one to three months of therapy; (3) delayed improvement with relief of ascites and edema occurring over six and eight month periods; and (4) failure to improve. Three patients were treated with a low sodium diet in conjunction with the albumin and are therefore classed separately.

Prompt diuresis occurred in four patients with complete relief of ascites and edema (Table I). This took place during or immediately following the administration of from 300 to 450 grams of albumin. Weight loss began in each instance before a sustained rise in serum albumin concentration was detected. The course of one patient typical of the group is shown in Figure 1. It can be seen that weight loss began in this patient before the serum albumin concentration rose above

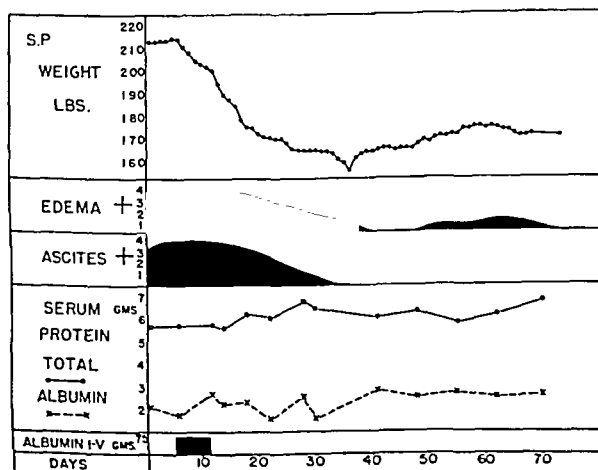


FIG. 1

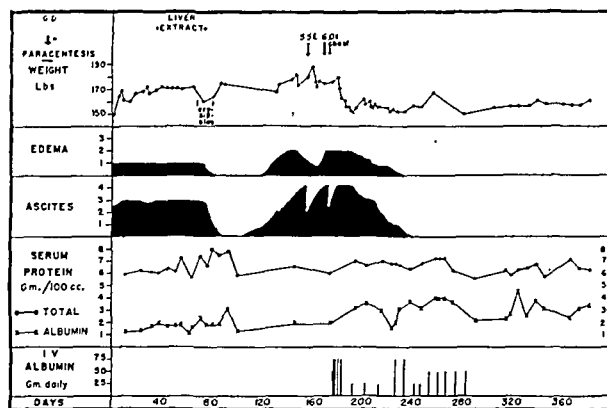


FIG. 2

3.0 grams per 100 cc. Edema and ascites recurred in one patient with this type of response six weeks after therapy was stopped. Improvement was apparently permanent in two others who have been followed for five and 11 months, respectively. The fourth patient had a fatal hemorrhage from esophageal varices one week after the last administration of albumin.

Definite improvement at a slower rate was seen in five patients after therapy had been continued for from eight days to three months (Table I). The response shown by this group is typified by the findings in one patient (G. D.) shown in Figure 2. Of these five patients, recurrence of ascites and edema took place in one (H. H.) approximately six weeks after the last injection of albumin. This patient was subsequently refractory to retreatment and died of hemorrhage from esophageal varices during therapy approximately five months after the initial albumin was given. A second patient (A. D.), although free of ascites for 11 months, noted a recurrence of peripheral edema six weeks after the cessation of albumin. Varicose veins in both lower extremities undoubtedly contribute to the edema in this patient. The improvement of the remaining three is maintained at eight, 10, and 16 months' follow-up examinations.

The two patients in whom disappearance of ascites and edema occurred only after prolonged treatment received albumin injections over periods of six and eight months, respectively (Table I). At the end of this time each had stopped reforming ascites in spite of having required numerous paracenteses before and during treatment.

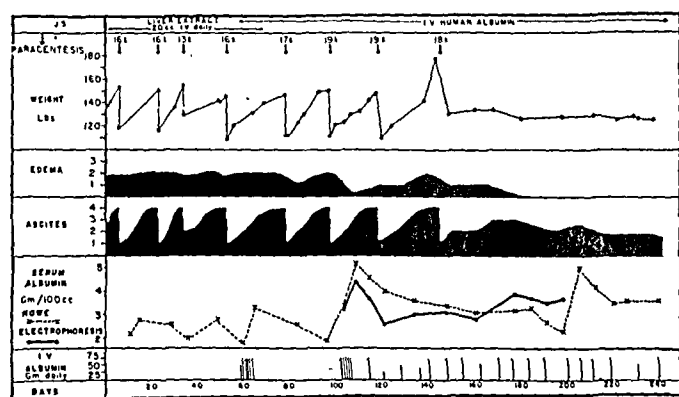


FIG. 3

One of these patients, J. S., seen six months after therapy was discontinued, showed no ascites in spite of the resumption of a large intake of alcohol, and the second has been seen eight months after cessation of albumin therapy, still free of ascites. The hospital course of the first of these is shown in Figure 3. It may be seen that electrophoretic analysis⁵ of the serum albumin concentration was generally lower than that determined by the Howe separation. Thus, the level at which the serum albumin was actually maintained was considerably lower than was supposed. Moreover, reaccumulation of fluid ceased coincident with a rise in serum albumin concentration (electrophoretic analysis) which was not associated with an increase in albumin administered. Whether this rise resulted from cessation of ascites formation and removal, or from increased production of albumin by the liver, cannot be ascertained from these data. The ineffectiveness of albumin in the amount administered is demonstrated.

Because of rapidly reforming ascites in spite of albumin administration, three patients were treated with low sodium diets⁶ while receiving maintenance injections of albumin (Table I). One of these was well controlled without paracentesis for two months while previously he had required paracentesis at two-week intervals. At the end of this period, five days after the last albumin administration, he bled from esophageal varices and

⁵ Per cent of the total serum protein (Kjeldahl) appearing as albumin in the Tiselius pattern. The authors express their appreciation to Dr. Frank H. Clarke, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for these determinations.

⁶ The low sodium diet provided approximately 100 grams protein and 3,000 calories but only 21 mEq. Na (12 grams NaCl) daily.

expired. A second patient was followed for eight months, during which time only one paracentesis, three months after the diet was begun, was required. Previous to the low sodium regimen, in spite of albumin therapy, paracentesis had been necessary twice a month. Figure 4 shows part of the course of the latter patient. The third patient, now under treatment for seven months, continues to reform ascites but at a considerably reduced rate while receiving a diet restricted in sodium. The importance of sodium in cirrhosis has been the subject of a separate investigation (8).

Despite continued therapy with albumin for from five days to four months, six of the 20 patients in whom the effect of albumin could be evaluated must be considered failures. During the period of treatment the attainment of significantly increased serum albumin concentrations did not improve ascites although edema of the lower extremities was decreased. The end results and reasons for cessation of therapy in this group of failures are shown in Table I. Reformation of ascites was accentuated in one patient by albumin administration, as shown in Figure 5. None of this group is still living.

An indeterminate group also included in Table I has been so classified because treatment could not be continued or the follow-up was insufficient to enable judgment of the result. None of these patients is now receiving albumin. Two may be considered to have received some benefit but other factors cannot be definitely ruled out. One patient, B. B., began to form ascites while recovering from jaundice after a prolonged bout of drinking. During the five-day period when albumin was given, weight loss occurred but this may have been

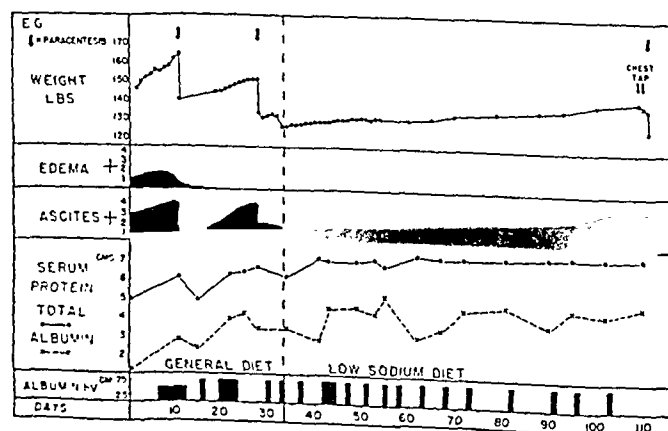


FIG. 4

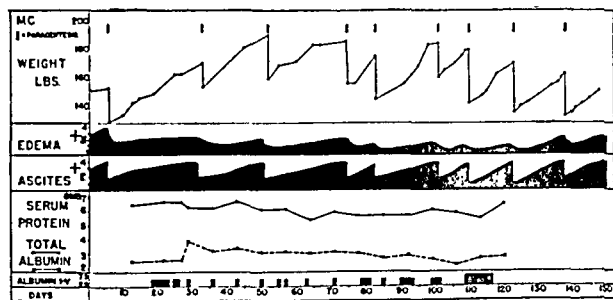


FIG. 5

spontaneous. The second, F. H., was a patient with known esophageal varices for which a spleno-renal anastomosis had been done. Post-operatively, ascites formation became apparent and albumin was therefore administered. Weight loss and disappearance of the peritoneal fluid took place but the factor of diminished portal pressure following the shunt procedure may have been operative in this case.

Untoward effects of albumin administration

The intravenous administration of concentrated albumin as a therapeutic agent in cases of hypoproteinemia has been accompanied by certain untoward reactions which should be emphasized. In this series of 29 cases, reactions which albumin may have precipitated were seen in ten. As mentioned above, fatal hemorrhage from esophageal varices occurred in three patients during the period of albumin therapy. One occurred one week after the last previous albumin infusion, one five days after, and the third patient had hematemesis six hours after an infusion which was pyrogenic. Although in cirrhosis with varices hemorrhage may occur at any time, albumin may have been a precipitating factor by increasing the plasma volume (9).

Hematemesis occurred in one patient after albumin administration, with recovery. Subsequently, a duodenal ulcer was demonstrated as the probable source of the bleeding. In a patient with frequent epistaxis prior to treatment, fatal hemorrhage from the nose took place after he had received 525 grams of albumin over an 18-day period. Such incidents as these two must also be considered possible sequelae of albumin therapy.

One patient (P. M.) with a previous history of cardiac failure during an attack of acute glomerulonephritis developed pulmonary edema during

the second daily infusion of 75 grams of albumin, although he showed no physical signs of cardiac disease at the beginning of treatment. He died in cardiac failure 12 hours thereafter. A 65-year-old female also developed pulmonary edema during the second day of albumin therapy (75 grams daily); albumin was discontinued and she recovered. Two patients, both elderly women, developed pleural effusion while receiving albumin and required thoracenteses. In the first (H. K.), severe dyspnea was noted after one week's administration and treatment was discontinued, while the second (E. G.) showed slowly progressive dyspnea in spite of the maintenance of a low sodium intake. Cessation of albumin therapy resulted in control of pleural fluid formation. With the exception of the first of these patients, P. M., no history or sign of cardiac disease had been noted prior to treatment and cessation of therapy was not followed by residual signs of heart disease. However, the dyspnea seen in patients H. K. and E. G. was due to the pleural effusion and probably was not of cardiac origin.

Pyrogenic reactions following albumin administration have been rare, and in no case has the albumin supplied been directly implicated. However, the dangers of such pyrogenic reactions in advanced cirrhosis are amply illustrated by the one fatality occurring under these circumstances. C. F., a 56-year-old female, received albumin for 12 days with what appeared to be a good response. On the 12th day she was given 50 grams of albumin mixed with liver extract and glucose solution. A severe chill and fever followed and hepatic coma ensued from which she did not recover. No instances of homologous serum hepatitis were observed following the intravenous administration of human serum albumin in this study.

Factors influencing the retention of albumin

In attempting to determine which patients with cirrhosis will show a diuresis following albumin, the number of paracenteses necessary, the effect of entry of albumin into the ascitic fluid, and the amounts of protein lost in ascitic fluid when paracentesis is necessary have been analyzed for the patients in this series. In addition, a study was carried out of the efficacy of a low sodium diet in reducing the transfer of albumin into ascitic fluid, and subsequent loss by paracentesis.

The relationship between the number of paracenteses necessary before albumin therapy and the response obtained after treatment is shown in Table I. As can be seen, the patients who had not yet reached the stage where paracentesis was absolutely necessary had an immediate diuresis when given albumin. In these subjects, the edema of lower extremities, penis and scrotum was massive while the ascitic fluid collection was not usually severely distressing. Comparing the groups who responded to therapy with those showing no response, only seven of the 11 patients who were improved had previously required one or more paracenteses while all of the patients who required the use of a low sodium diet or who failed to improve had had paracentesis prior to treatment. If, as is generally accepted, the necessity for abdom-

inal fluid removal is an indication of the severity of liver disease, then it is apparent that those patients whose disease is more severe have the poorest therapeutic result when albumin is given.

Mankin has shown that the osmotic pressure changes of ascitic fluid and serum parallel each other under varying conditions in patients with liver disease and ascites (10). The protein values of serum and ascitic fluid obtained from 15 patients in this series, before and during albumin therapy are listed in Table II. The total protein concentration in the ascitic fluid rose in all patients as albumin was administered intravenously. Moreover, this occurred in patients F. F. and J. G., both of whom had a prompt diuresis with disappearance of ascites and edema. The protein content of edema fluid (obtained by Southey tubes)

TABLE II
Changes in protein concentration of ascitic fluid and serum during albumin administration

Patient	Total albumin given grams	Interval between determinations days	Serum total protein grams/100 cc.		Serum albumin grams/100 cc.		Ascitic fluid total protein grams/100 cc.	
			Before therapy	During therapy	Before therapy	During therapy	Before therapy	During therapy
Immediate improvement								
F. F.	375	5	5.8	6.1	1.8	3.6	0.6	2.0
J. G.	400	4	6.3	5.8	2.0	3.1	0.8	2.0
Slow improvement								
G. D.	425	6	6.5	7.0	2.7	3.9	0.8	2.6
P. B.	375	9	6.7	7.0	2.2	4.3	1.2	3.6
J. M.	450	8	5.3	7.0	2.3	4.1	*	4.0
H. H.	300	6	6.4	7.3	1.9	4.1	0.5	2.1
Delayed improvement								
J. S.	450	15	6.5	6.2	3.2	3.7	1.3	2.4
W. M.	300	25	4.7	6.8	1.7	2.8	1.6	3.1
Improved only with low sodium diet								
E. G.	300	9	4.9	6.1	1.2	2.8	*	2.9
V. L.	300	9	6.6	7.4	2.1	3.1	*	2.2
C. M.	825	16	6.7	6.9	1.5	4.6	1.8†	4.4
Failure								
M. C.	350	30	6.8	6.2	2.5	3.2	*	2.2
H. G.	975	25	7.8	7.5	2.6	2.7	*	3.2
N. C.	825	16	6.9	7.4	2.9	3.7	1.0	3.7
P. W.	1200	55	6.9	6.6	2.8	3.4	*	2.7

* Determination not done.

† Two days after albumin started.

TABLE III

Changes in protein concentration of edema fluid, ascitic fluid, and serum in three patients with prompt diuresis during albumin administration

Patient	Albumin administered between determinations	Day of study	Serum		Ascitic fluid		Edema fluid	
			Total protein	Albumin	Total protein	Albumin	Total protein	Albumin
	grams		grams/100 cc.	grams/100 cc.	grams/100 cc.	grams/100 cc.	grams/100 cc.	grams/100 cc.
F. F.	0	0	5.8	1.8	0.6	0.0	0.1	0.0
	75	1	6.1	1.5	0.7	0.0	0.2	0.0
	75	2	5.8	2.0	1.1	0.3	0.2	0.0
	75	3	5.7	3.2	1.2	0.4	1.0	0.3
	75	4	5.4	2.3	2.1	0.5	0.8	0.3
	75	5	6.1	3.6	2.0	1.4	1.1	0.6
J. G.	0	0	6.3	2.0	0.8	0.3	0.2	0.0
	100	1	6.2	1.6	0.9	0.3	0.4	0.0
	100	2	6.0	2.5	0.9	0.3	1.0	0.2
	100	3	6.6	2.5	1.9	0.5	1.4	*
	100	4	5.8	3.1	2.0	0.5	*	*
S. P.	0	0	5.7	1.7	0.8	0.0	0.2	*
	450	11	6.3	2.3	*	*	0.7	*

* Determination not done.

from these two patients and from a third, S. P., also rose during treatment (Table III, Figure 6).⁷ It is apparent that the entry of protein into the ascitic fluid occurred regardless of the final result and that a rise in protein content of edema fluid did not interfere with its reabsorption.

The entry of protein into the ascitic fluid is a source of protein loss in patients with cirrhosis because of repeated removal by paracentesis.⁸ The

total protein lost in ascitic fluid removed was compared in five patients with the total amount of albumin administered since the previous paracentesis. The results of this "balance" between parenteral protein administered and ascitic fluid protein removed are shown in Table IV. While it is realized that this is but a rough estimate and is subject to many variables, it may be seen that the total loss of protein by entry into the ascitic

⁷ A variable and undetermined portion of the increase in concentration of protein in the edema fluid and ascitic fluid of patients undergoing rapid diuresis may result from more prompt reabsorption and mobilization of the water and electrolytes than of the protein. It is impossible to ascertain, therefore, to what extent the intravenously administered albumin contributed to the final concentration by its entry into these fluids.

⁸ Albumin administered intravenously does not usually appear in the urine as whole protein. Although a large portion of injected albumin may be excreted into the urine of patients with preexisting renal disease, only a negligible amount escapes through the normal kidney even after several days of repeated daily infusions. Determinations for protein were made in the urine of several of the patients in this study. A total proteinuria of 5 to 10 grams was occasionally noted during the initial period when approximately 500 grams of albumin were administered within a week to raise the serum albumin concentration to normal, but was not subsequently observed while maintenance injections of approximately 75 grams weekly were required to maintain the serum albumin concentration. Thus, the loss of albumin into the urine of the patients reported in this study did not constitute a significant loss of administered protein.

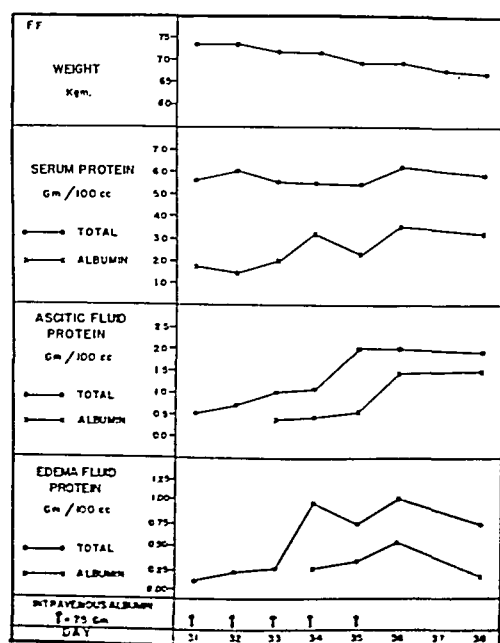


FIG. 6

TABLE IV
Protein loss in ascitic fluid during albumin administration

Patient	Interval between paracenteses	Fluid removed	Protein concentration of ascitic fluid	Total protein lost	Albumin given between paracenteses
	days	liters	grams/100 cc.	grams	grams
J. S.	19	15.0	1.3	195	425
	19	10.5	1.8	189	0
	22	18.5	2.4	444	450
	27	18.0	1.6	288	200
	Total			1116	1075
W. M.	18	16.0	3.1	496	250
	18	13.0	2.2	286	175
	9	9.0	3.0*	270	450
	37	13.0	4.1	533	375
	40	14.0	4.0*	560	675
	49	14.0	4.1	574	1125
	Total			2719	3050
H. H.	12	9.0	1.7	153	150
	33	10.1	3.2	323	800
	12	10.5	2.3	242	150
	Total			718	1100
M. C.	7	8.0	2.2	176	350
	21	14.0	2.0	280	150
	11	8.2	2.3*	189	200
	21	15.0	2.6	390	200
	17	12.0	2.7*	324	400
	9	17.5	2.8	490	375
	Total			1849	1675
P. W.	6	7.5	2.0*	150	450
	49	11.0	2.7	297	750
	17	15.0	2.5*	375	375
	11	15.0	3.0	450	450
	21	15.0	2.3	345	325
	Total			1617	2350

* Estimated—determination not done.

fluid during albumin therapy is large and may even surpass the amount of protein given intravenously as albumin.

The formation of ascites was not improved in three patients in this series by the intravenous administration of albumin until a low sodium diet was simultaneously provided (Table I). It was observed that reducing the sodium intake slowed the formation of ascites, prolonged the period between paracenteses, and thus diminished protein loss. Table V shows the findings in two of the patients given albumin to maintain normal serum albumin concentrations. Ascitic fluid was collected when paracentesis became necessary, both after a period of high salt intake and again after a period of low salt intake. Although the protein loss in ascitic fluid was not markedly changed in quantity at any one paracentesis, it is apparent that the period between paracenteses was prolonged by the low sodium diet and that less albumin per day was required to maintain the serum albumin at normal concentrations during the low sodium period.

Changes in liver function tests following albumin administration

The effect of albumin administration on three liver function tests is shown in Table VI. Serum bilirubin and thymol turbidity were determined at regular intervals whereas bromsulphalein retention was done less frequently. The results were extremely variable and no consistent picture was shown. It has been demonstrated (11, 12) that

TABLE V
Conservation of albumin with low sodium diet

	E. G.		C. M.	
	Unrestricted salt intake	Low sodium intake	Unrestricted salt intake	Low sodium intake
Interval between paracenteses (days)	23	75	29	39
Total albumin given intravenously (grams)	525	1125	975	525
Average albumin given intravenously daily (grams)	22.8	15.0	33.6	13.5
Protein removed in ascitic fluid (grams)	300 (T.P. 3.0 grams %) (10.0 liters)	258 (T.P. 3.0 grams %) (8.6 liters)	444 (T.P. 3.7 grams %) (12.0 liters)	468 (T.P. 3.1 grams %) (15.1 liters)

TABLE VI
Liver function tests before and after albumin administration*

Patient	Interval between determinations months	Total serum bilirubin mgm./100 cc.		Thymol turbidity cc. BaSO ₄		Bromsulphalein retention %	
		Before therapy	After therapy	Before therapy	After therapy	Before therapy	After therapy
Immediate improvement							
S. P.	1	7.0	2.7	1.1	2.2	31	25
F. F.	1	3.9	2.4	2.5	1.8		
J. G.	1	16.2	8.3	3.3	3.0		
Slow improvement							
G. D.	3	6.5	4.0	3.1	2.1	16 42	42 16
P. B.	2	3.8	1.2	4.4	3.7		
J. M.	2	0.4	0.8	1.3	2.6		
A. D.	2	2.8	4.3	4.2	1.6		
H. H.	1	6.0	3.8	3.0	2.4		
Delayed improvement							
J. S.	6	0.3	1.4	1.5	1.7	32	32
W. M.	8	0.4	1.1	2.4	2.3	24	18
Improved only with low sodium diet							
E. G.	8	1.4	2.7	1.2	1.8	43	36
V. L.	2	0.4	0.5	2.5	2.5		
C. M.	7	1.0	1.5	4.7	1.7		
Failure							
M. C.	4	0.6	1.2	2.1	0.6	36	48
P. W.	5	3.7	1.9	3.6	1.8	36	52

* Normal values: B.S.P.—Less than 5% retention (5 mgm. dye per kilogram body weight). Bilirubin—Less than 1.0 mgm./100 cc. Thymol Turbidity—Less than 1.7 cc. BaSO₄

serum albumin will alter the thymol turbidity. Therefore, the apparent improvement in this test shown by some patients is not reliable nor indicative of the true state of liver function. There was a tendency, however, for the serum bilirubin concentration to decrease in those patients who had a prompt diuresis with loss of ascites and edema. It can also be seen that some patients with nearly normal bilirubin before treatment did not show improvement in fluid retention. In the patients on whom bromsulphalein tests were done before and after treatment no significant change appeared even though loss of edema and ascites was effected by treatment in some.

DISCUSSION

The use of concentrated albumin in the treatment of edema and ascites associated with hypoal-

buminemia has been viewed with optimism on theoretical grounds. Encouraging results were obtained by Kunkel (3) early in the study of its use in cirrhosis of the liver but the results of Thorn *et al.* (2), and Patek *et al.* (13) have borne out the original conservative estimate of its value made by Janeway and his co-workers (1). The series reported here reveals that a wide range of results may be obtained even when patients are treated over long periods of time. Although striking improvement was seen in a few patients, the relatively poor effect in 11 out of 20 patients studied (not including nine patients in whom the effect of albumin could not be satisfactorily evaluated) leads to a similar conservative estimate of the efficacy of albumin therapy in chronic liver disease. The fact that some patients in our series improved after the prolonged administration of

albumin suggests that such improvement may have been the result of dietary treatment alone. Furthermore, since it has been shown that albumin administered intravenously can be utilized as a protein nutrient (14), some benefit was undoubtedly derived from this nutrient effect of albumin. However, albumin is neither a specific for the treatment of the fluid retention nor the underlying liver disease.

The most economical use of albumin is obtained when the diet is low in sodium, since the formation of ascitic fluid and loss of protein is greatly reduced by this means. This is in disagreement with Armstrong (15) who has minimized the importance of a low sodium intake in the treatment of liver disease. As the data presented here indicate, the removal of ascitic fluid containing 300 grams of protein may involve the waste of between 100 and 200 grams of albumin. A high intake of salt and water increases the formation of ascites. If ascitic fluid is present in large quantities or is rapidly forming, the entry of albumin into the fluid drains protein from the serum in larger quantities than would be the case were ascitic fluid present in small quantities or its formation diminished by measures such as sodium restriction. Furthermore, to maintain a given serum albumin concentration, less albumin is required when its loss into ascitic fluid is minimized by reducing the rate of formation of ascites. A low sodium diet is of most benefit to those patients who require repeated paracenteses. However, when albumin therapy is being initiated in a case of anasarca, a diet low in sodium should yield the most efficient use of albumin, regardless of the final response.

The mechanism by which concentrated albumin induces a diuresis is not clear. Albumin administration undoubtedly increases the serum osmotic pressure. Evidence suggesting that this is an important factor is the observation of Thorn *et al.* (2) and substantiated here, that peripheral edema is most easily mobilized when therapy is begun. The importance of the osmotic factor is minimized, however, by the finding of increased concentrations of protein in the edema and ascitic fluid when albumin is given intravenously. The increase in ascitic fluid protein thus produced would appear to emphasize the importance of portal hypertension as a causative factor in ascites formation. It should be pointed out, however, that the passage of pro-

tein into ascitic fluid following intravenous albumin took place even in patients undergoing a rapid diuresis. It seems likely that factors in addition to lowered serum osmotic pressure and portal hypertension are operative in the retention of fluid in cirrhosis of the liver. Patek has shown in a limited series of studies that there is increased renal blood flow and glomerular filtration when albumin is administered (13), presumably resulting from the increased plasma volume. This may hasten salt and water excretion by the kidney. Further studies of renal function in cirrhosis both before and during albumin therapy are clearly indicated.

The occurrence of harmful effects from albumin therapy must be emphasized. Since a majority of patients with cirrhosis are over the age of 45, and are therefore subject to possible degenerative heart disease, it may be expected that a rapid increase in plasma volume such as is produced by albumin may result in cardiac complications.

Finally, the augmented plasma volume following albumin administration may increase the burden upon esophageal varices and precipitate rupture and hemorrhage. Such an occurrence may have been the cause of death in three patients in this series. In view of such incidents albumin should be administered cautiously and heroic efforts to raise serum albumin levels above normal are to be avoided.

SUMMARY AND CONCLUSIONS

1. Concentrated salt-poor human serum albumin was administered intravenously to 29 patients with cirrhosis of the liver who had fluid retention. The albumin was administered over periods varying from four days to eight months. The data were considered suitable for analysis in 20 of the patients. Of these, four patients had an immediate diuresis with loss of ascites and edema, five had a slow response in from one to three months, and two recovered from ascites and edema after prolonged treatment of six and eight months, respectively. Nine patients must be considered failures although the fluid retention was partially controlled in three by the use of a low sodium diet. The four patients who underwent immediate improvement had not required paracentesis before albumin therapy and had massive edema. Diuresis might have occurred subsequently in these pa-

tients from a nutritious diet alone. Nevertheless, albumin administration was effective by inducing an immediate, rapid diuresis, beginning before the serum albumin concentration reached normal. The slow response in seven patients cannot be definitely attributed to the albumin administered as the other therapeutic measures used concomitantly might have given as satisfactory results.

2. Fatal hemorrhage from esophageal varices was coincident with, or shortly followed, albumin administration in three patients. Severe epistaxis and bleeding from a duodenal ulcer may have been induced by this therapeutic measure. Pulmonary edema and pleural effusions were observed as untoward effects, presumably attributable to albumin therapy. The use of this form of therapy, especially in elderly or cardiac patients, or in the presence of known esophageal varices, is dangerous and caution should be exercised.

3. Albumin entered the ascitic and edema fluid following its intravenous infusion whether diuresis occurred or not. The total protein content of the ascitic fluid rose in approximately parallel fashion with the rise in serum albumin. During a given period between paracenteses in five patients the protein lost in the ascitic fluid approximated the amount of albumin administered intravenously.

4. Ascitic fluid formation was found to be retarded by a low sodium diet in three patients, and thus the loss of albumin by paracentesis was reduced. Sodium restriction allowed the maintenance of normal serum albumin concentrations more economically than did a diet unrestricted in sodium.

5. Serial serum bilirubin, thymol turbidity and bromsulphalein tests showed no consistent change during or after treatment with albumin. Recovery from ascites and edema was seen to precede the return of liver function tests to normal values.

6. It is concluded that concentrated salt-poor human serum albumin, under the conditions of this study, is neither specific for the relief of ascites nor markedly beneficial in the treatment of the underlying liver disease. In view of the variety of results obtained with concentrated human serum albumin in patients with cirrhosis of the liver, it is concluded that other factors in addition to a reduced serum osmotic pressure, as

represented by decreased serum albumin concentrations, are active in the retention of fluid.

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THE EFFECT OF HUMAN SERUM ALBUMIN, MERCURIAL DIURETICS, AND A LOW SODIUM DIET ON SODIUM EXCRETION IN PATIENTS WITH CIRRHOSIS OF THE LIVER^{1, 2, 3}

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The relative importance of the serum albumin concentration, portal hypertension, and antidiuretic substances in the formation of ascites and edema in patients with cirrhosis of the liver has not been clarified. There is evidence, however, that each of these factors is important (1-3).

In an effort to eliminate the factor of hypoalbuminemia, salt-poor concentrated human serum albumin has been given intravenously. Although the serum albumin concentration may by this means be raised to normal, the edema and ascites have not been consistently relieved (4-7). Moreover, improvement in ascites and edema is observed in some instances with mercurial diuretics or simply by furnishing a nutritious diet alone (8, 9). In an attempt to find an abnormality common to these patients and consistently altered by the apparently unrelated therapeutic agents, the studies of sodium excretion and balance reported here were undertaken.

METHODS

The 11 patients studied had ascites and edema associated with cirrhosis of the liver, established by history,

¹ A preliminary report of this investigation was presented before the national meeting of the American Federation for Clinical Research, Atlantic City, N. J., May 4, 1948 (*Am. J. Med.*, 1948, 5, 623).

² The serum albumin used in this study was processed by the American National Red Cross from blood which it collected from voluntary donors. This is one of a series of investigations on serum albumin being carried out with material supplied by the American National Red Cross. As soon as sufficient data become available to justify final conclusions concerning its therapeutic value a full report to the medical profession on the use of serum albumin in medical practice will be published.

³ This study was aided in part by a gift to Harvard University from the Abbott Laboratories, North Chicago, Illinois.

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physical examination, liver function tests, and occasionally by needle biopsy. Six patients had had a previous trial with intravenous albumin when the diet was unrestricted in sodium without marked response (patients V. L., W. M., C. M., H. H., A. McM., and J. McC.). The others were observed for two or more weeks on an unrestricted diet and no tendency for spontaneous improvement had been shown at the time studies were begun. None of the patients showed evidence of co-existing cardiac or renal disease.

With the exception of experiment No. II (S. P.), a low sodium diet was used. To increase the protein and caloric content of the diet, 150 grams of salt-poor milk powder containing 45 grams of protein mixed with water were used in place of milk.⁶ By calculation,⁷ the diet contained 100 grams of protein, 3,000 calories, and only 21 mEq. (0.5 grams) of sodium daily, and was well tolerated by the subjects. The sodium contained in the albumin solution⁸ was added to the sodium intake. Patients who received added sodium were given weighed amounts of salt and were either instructed to use it daily on their food or it was mixed in food and fluids consumed.

All patients were carefully followed on the Thorndike metabolic ward, with nurses and a dietitian in constant attendance. Strict control of each patient's dietary intake and urinary output was maintained throughout the study. All patients were allowed to drink water freely, and the daily fluid (liquid) intake was recorded. Fluid balance measurements obtained by differences between the fluid intake (liquids plus estimated fluid ingested in the diet) and fluid output (urine plus estimated fluid lost via the

⁶ "Lonalac," supplied by Mead Johnson and Company, Evansville, Indiana. Each 150 grams of powder contains approximately 0.7 mEq. (15 mgm.) of sodium.

⁷ It has been pointed out (10) that diets analyzed for sodium usually contain more than the amount calculated. This, in addition to the fact that strict control over the use of canned foods was not always possible, constitutes probable sources of error in our calculation of sodium balances. As will be seen, however, these factors merely accentuate the retention of sodium shown in these patients. Thus, when sodium equilibrium is shown, there is probably in reality a slight positive sodium balance.

⁸ The sodium contents of the lots of albumin used were obtained through the courtesy of Dr. Charles A. Janeway, Children's Hospital, Boston. Each 25 grams of albumin contains approximately 15 mEq. (0.3 gram) of sodium.

stools, lungs, and skin) are variable and unreliable. Since all patients in this study were weighed daily under similar conditions, it was considered that changes in body weight reflected more accurately changes in fluid retention in the body than did the difference between the fluid intake and the urine output.

Urine sodium excretion was determined daily except for a few studies in which the urine was analyzed in three- or four-day pools. The method of Consolazio and Dill was used (11). The extrarenal sodium loss was estimated to be constant at 20 mEq. per day (12-14). The studies were carried out during the winter months so that perspiration was minimal.

Concentrated salt-poor human serum albumin (25 per cent solution) was given in amounts of from 25 to 75 grams (100-300 cc.) in each injection with equal volumes of 5 or 10 per cent dextrose in distilled water. When maintenance of the serum albumin concentration was desired, 25 grams of albumin were administered daily, or three times weekly. The serum albumin concentration was determined by micro-Kjeldahl analysis of fractions separated by the method of Howe (15).

RESULTS

I. The effect on ascites and edema of varying the salt intake while maintaining a normal serum albumin concentration (Table I, Figures 1-5)

Four patients (W. M., C. M., H. H., and V. L.) who had previously had numerous abdominal

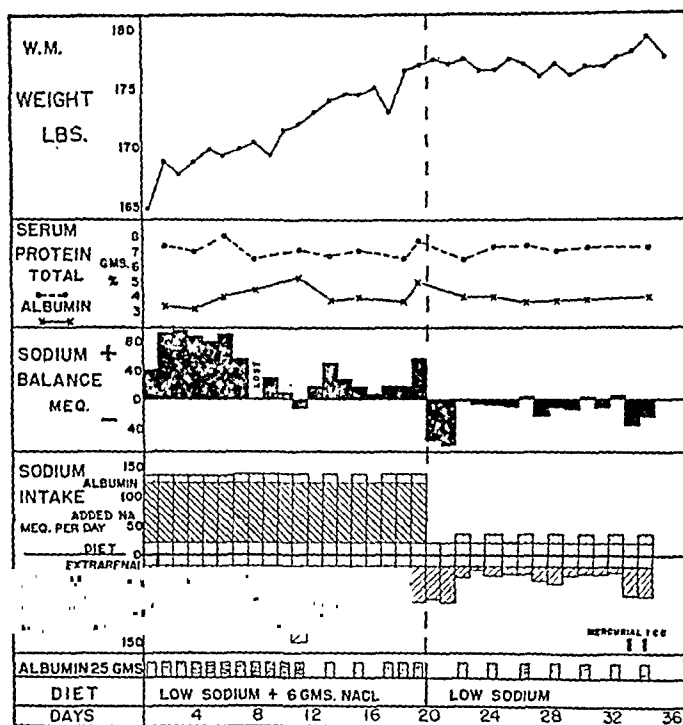


FIG. 1

paracenteses with regular reaccumulation of fluid, the "stabilized cirrhosis" of Patek (16), were maintained on the low sodium diet (21 mEq. daily). They were then given sufficient albumin

TABLE I

Sodium excretion following intravenous saline

Low sodium (21 mEq.) diet throughout all studies. Serum albumin concentrations maintained within normal limits.

Day	Solution administered	C. M.			A. McM.			G. C.*		
		Sodium intake	Sodium output urine	Sodium output extrarenal†	Sodium intake	Sodium output urine	Sodium output extrarenal†	Sodium intake	Sodium output urine	Sodium output extrarenal†
		mEq./day	mEq./day	mEq./day	mEq./day	mEq./day	mEq./day	mEq./day	mEq./day	mEq./day
1	—	21	2.0	20	21	23.7	20	21	3.8	20
2	0.85% saline 1,000 cc.	165	6.8	20	165	66.9	20	165	87.2	20
3	Alb. 25 grams	36	12.0	20	36	29.5	20	36	73.8	20
4	5% saline 170 cc.	165	1.9	20	165	44.4	20	165	126.4	20
5	Alb. 25 grams	36	1.7	20	36	56.7	20	36	10.5†	20
6	—	21	2.6	20	21	20.5	20	21	70.7	20
Total sodium mEq./6 days		444	147		444	362		444	492	
Sodium balance mEq./6 days		+297			+82			-48		
Theoretical weight change§ (lbs./6 days)		+4½			+1½			-¾		
Actual weight change (lbs./6 days)		+5			+½			-2		

* Patient undergoing diuresis following albumin therapy.

† Estimated.

‡ Incomplete collection.

§ Calculated from the sodium balance, each 140 mEq. Na estimated to represent 1 liter of water (1 Kgm. or 2.2 lbs.)

intravenously to raise their serum albumin concentrations to above 3.2 grams per 100 cc. This, or a higher concentration, was maintained throughout the period of study. After a suitable control period on the low sodium intake, each patient was given daily 6 grams of salt (102 mEq. of sodium). Changes in sodium balance and weight in three of the patients are shown in Figures 1-3. Weight changes reflecting the probable alterations in sodium balance on the fourth subject are shown in Figure 4. In all four patients there was a similar

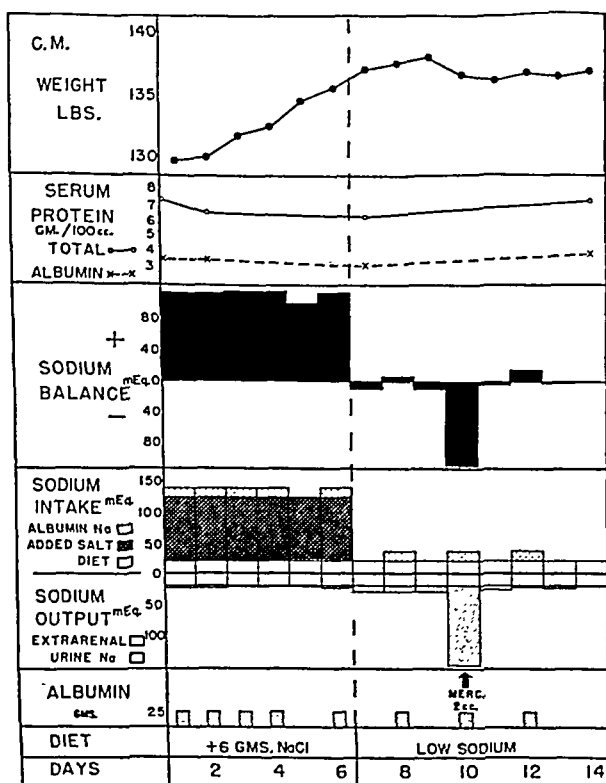


FIG. 2

increase in weight and ascites formation coincident with the administration of added salt. During this period the excretion of sodium in the urine of two patients (C. M. and H. H.) was no greater than during the period of low sodium intake, and was only moderately increased in the third patient (W. M.). As a result, all patients were in a markedly positive sodium balance while receiving supplemental sodium salts. Thus, a normal serum albumin concentration does not alone permit the excretion of excess sodium given orally.

It seemed possible that patients with cirrhosis

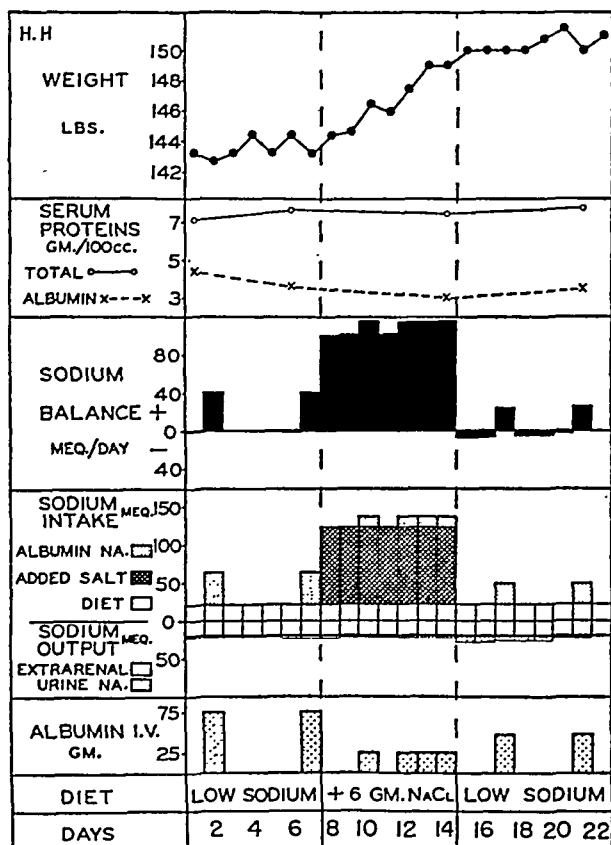


FIG. 3

of the liver might react differently to sodium administered intravenously. To study this, two patients, maintained on a low sodium diet, were given sufficient albumin to attain normal serum albumin concentrations and were then given saline intravenously in varying concentrations (C. M. and A. McM., Table I and Figure 5). After a control day, 1,000 cc. of isotonic saline was given intra-

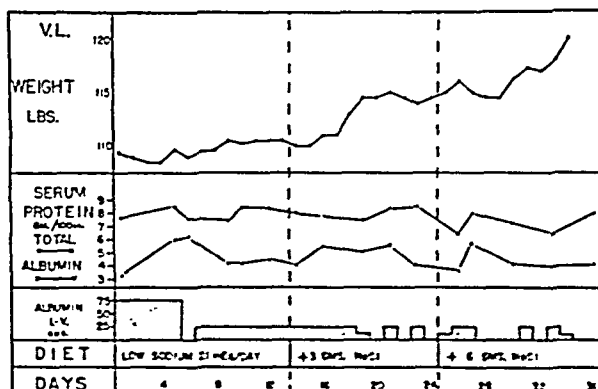


FIG. 4

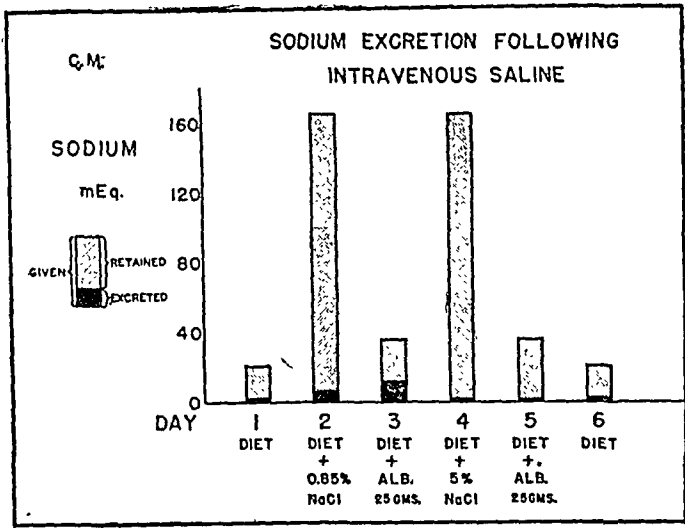


FIG. 5

venously. The following day, 25 grams of albumin were given and on the fourth day 170 cc. of hypertonic (5 per cent) saline. On the fifth day the albumin was repeated. A marked inability to excrete sodium was noted in both patients following the intravenous administration of isotonic and hypertonic saline. Moreover, diuresis did not occur following the saline administration.⁹ Although patient A. McM. was able to excrete sodium in a more nearly normal manner than C. M., neither subject was able to excrete sodium in high concentrations. Thus, the degree of sodium excretion varies with individual patients. These findings may be contrasted with those of a similar study on a patient with cirrhosis undergoing diuresis after serum albumin therapy (G. C., Table I). The latter was able to achieve sodium equilibrium.

II. The effect of albumin administration on the excretion of sodium (Table II, Figure 6)

Prompt diuresis with rapid loss of both edema and ascites is seen in a few patients with cirrhosis of the liver following the intravenous administra-

⁹ The administration of hypertonic salt solution intravenously in normal individuals stimulates the release of an antidiuretic substance from the pituitary and diminishes urine flow for short periods of time (17). However, the administration of saline in hypo-, iso-, or hypertonic concentrations in normally hydrated individuals, allowed free access of water, results in a considerably increased excretion of water if the urine collections are made over longer periods of time, as in the present study (18).

tion of albumin (7). One such patient (S. P., Figure 6) with marked ascites and massive edema of the lower extremities, scrotum and penis, was selected for study of sodium excretion. He was maintained on a diet calculated to contain 91 mEq. of sodium daily. During the six days prior to the administration of albumin his urinary excretion of sodium averaged only 5 mEq. daily. On the second day of albumin administration (75 grams daily for six days) his urinary sodium excretion rose to 236 mEq. and thereafter averaged 156 mEq. daily. Weight loss began on the first day of albumin administration, and diuresis was sustained until he became free of ascites and edema after 25 days.

Such dramatic results following albumin administration are seen in only a few patients. More commonly, a temporary diuresis and loss of weight follow, or merely a decrease in the rate of weight gain may be observed. A patient (C. M., Table II) whose response fell into the latter category was provided with the low sodium diet to which, after a short control period, 6 grams of salt (102 mEq. of sodium) were added. With this high salt regimen he gained weight rapidly and sodium excretion remained at the control value of about 4 mEq. daily, so that he was in a marked positive sodium balance. Beginning on the sixth day he was given 50 grams of albumin daily for six days and 25 grams daily thereafter. Urinary sodium rose to 21 mEq. daily in the 12th to 14th day pool and subsequently to 40 mEq. daily in the next three-day pool as the serum albumin concentration further increased. However, sodium equilibrium was not achieved, and ascitic fluid accumulation continued. In spite of omitting the added salt for five days, with apparent negative sodium

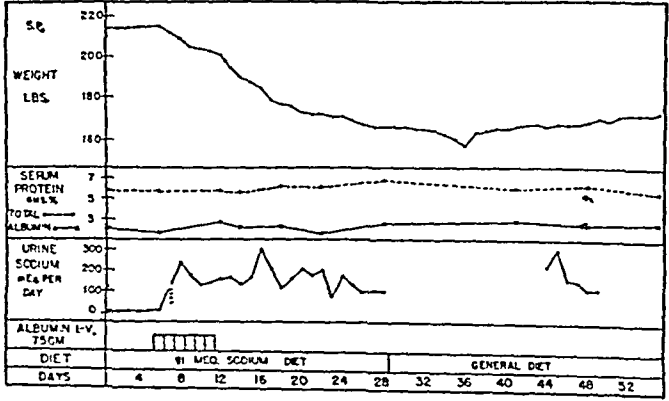


FIG. 6

TABLE II
Sodium excretion following intravenous albumin

Day	C. M. 123 mEq. sodium diet		J. McC. 123 mEq. sodium diet		H. H. 21 mEq. sodium diet	
	Albumin administered*	Urine sodium	Albumin administered*	Urine sodium	Albumin administered*	Urine sodium
	grams	mEq./day	grams	mEq./day	grams	mEq./day
1		4.3		2.7		1.0
2		4.0		6.0		1.3
3		5.1		18.0		1.8
4		7.5		19.4	75	4.2
5		2.5		17.5	Paracentesis	1.1
6	50	4.3	50	35.5	75	3.3
7	50	—	50	31.4	75	4.5
8	50	—	50	42.9	75	6.2
9	50	4.9	50	93.4	75	7.7
10	50	5.3	50	64.6	75	9.5
11	50	3.2	50	64.6	75	3.0
12	—	21.0	50	64.6		3.0
13	25	21.0		59.0		
14	25	21.0	25	59.0		
15	25	40.5				
16	25	40.5				
17	25	40.5				

* The administration of each 25 grams of albumin entails the giving of approximately 15 mEq. of sodium in addition to that in the diet.

balance during this period, a paracentesis became necessary.

Another patient (J. McC., Table II) with recurrent ascites and edema was similarly studied before and during the period of albumin administration. In the control period before albumin administration he was maintained on the low sodium diet during which time urinary sodium averaged 4 mEq. per day. Six grams of salt (102 mEq. sodium) was then added to the diet and the urinary sodium rose in the subsequent five days to an average of 13 mEq. daily with a maximum of 19 mEq. on the fourth day. In the next period 50 grams of albumin were given daily, intravenously. In this period there was an increase in urinary sodium to 35 mEq. on the first day and an average excretion of 57 mEq. per day for the nine days of albumin administration. The increased sodium excretion was followed by a slow diuresis.

A similar study was conducted with patient H. H. (Table II) who also had recurrent ascites. In his case, the low sodium diet was continued throughout without a period of added salt. After a control period of two days, albumin (75 grams daily) was administered, intravenously, for two days and on the third day ascitic fluid was removed by paracentesis. Albumin administration was then resumed for six more days. During the two-day

control period, urinary sodium was 1.0 and 1.3 mEq. daily, rising, after paracentesis, so that on the eighth day of albumin administration it was 9.5 mEq. Positive sodium balance and weight gain occurred during this period in spite of the increase in urinary sodium, since the administration of each 25 grams of albumin entails giving approximately 15 mEq. of sodium.

Data on the three patients shown in Table II demonstrate a considerable variability between patients in the increase of sodium excretion after albumin administration. A negative sodium balance was not achieved in these three patients in spite of albumin administration and the subsequent rise to normal of the serum albumin concentration.

III. The effect of mercurial diuretics on sodium excretion (Table III)

The rate of disappearance of ascites and edema following mercurial diuretics is often as rapid as after albumin administration. To compare the effect of mercurial diuretics on sodium excretion with the effect of albumin, two patients were studied. The first (J. Q.) had massive ascites and edema and a low serum albumin concentration. After a control period on a low sodium diet, he

TABLE III
Sodium excretion following intravenous mercurials
Low sodium diet—(21 mEq.)

J. Q. (serum albumin 1.8 grams %)			Day	C. M. (serum albumin 2.4 grams %)		
Urine sodium	Mercurial*	Weight		Urine sodium	Mercurial*	Weight
mEq./day		lbs.		mEq./day		lbs.
11.6		214 $\frac{3}{4}$	1	1.1		146 $\frac{1}{2}$
7.4		214 $\frac{3}{4}$	2	6.6		147
10.2		215 $\frac{3}{4}$	3	1.3		147 $\frac{3}{4}$
48.1	X	214 $\frac{1}{2}$	4	62.0	X	148
159.3	X	213 $\frac{1}{2}$	5	2.0		149
191.5	X	216	6	39.0	X	148
132.3	X	212 $\frac{1}{2}$	7	6.3		147 $\frac{1}{2}$
124.5	X	211 $\frac{3}{4}$	8	25.2	X	148
234.5		204 $\frac{1}{2}$	9	2.7		148
138.3		201 $\frac{1}{4}$	10	30.2	X	149 $\frac{1}{2}$

* 2 cc. "Mercuzanthin" intravenously as indicated (X).

was given 2 cc. of a mercurial diuretic,¹⁰ intravenously daily for five days (Table III). Urinary sodium excretion averaged 9 mEq. daily during the control period, rose to 48 mEq. on the first day of diuretic therapy, and thereafter averaged over 130 mEq. for 12 days. During this period he lost 20 lbs. of weight, and subsequently became free of ascites and edema.

A second patient (C. M.) with chronic, recurrent ascites, and whose serum albumin concentration was low, was given a low sodium diet. Mercurial diuretics were given every other day for eight days, and daily urinary sodium determined (Table III). On the days of mercurial administration, sodium excretion varied between 25 and 62 mEq., while on control days, both between mercurial injections and after they had been discontinued, urinary sodium never exceeded 10 mEq. The patient's weight changed little, however, and the marked diuresis seen in the first study did not occur.

Data on the patients shown in Table III demonstrate that the effect of mercurial diuretics varies in different patients and is possibly related to the relative severity of the liver disease.

IV. Sodium balance in a patient recovering from ascites (Figure 7)

In one patient (W. M.) whose tendency to re-form ascites appeared to be overcome, studies were

¹⁰ "Mercuzanthin," Campbell Products, Inc., New York, N. Y.

made of his ability to excrete sodium during the recovery phase. Following a two-month interval, during which he was given maintenance albumin and a general diet unrestricted in sodium, and had had one paracentesis, W. M. was restudied (Figure 7). At this time he was given the low sodium diet and mercurial injections with slight weight loss. On being given the 6 grams of added salt he was able to excrete over 100 mEq. of sodium daily in his urine, sufficient to achieve sodium equilibrium. Very slight weight gain in the first four

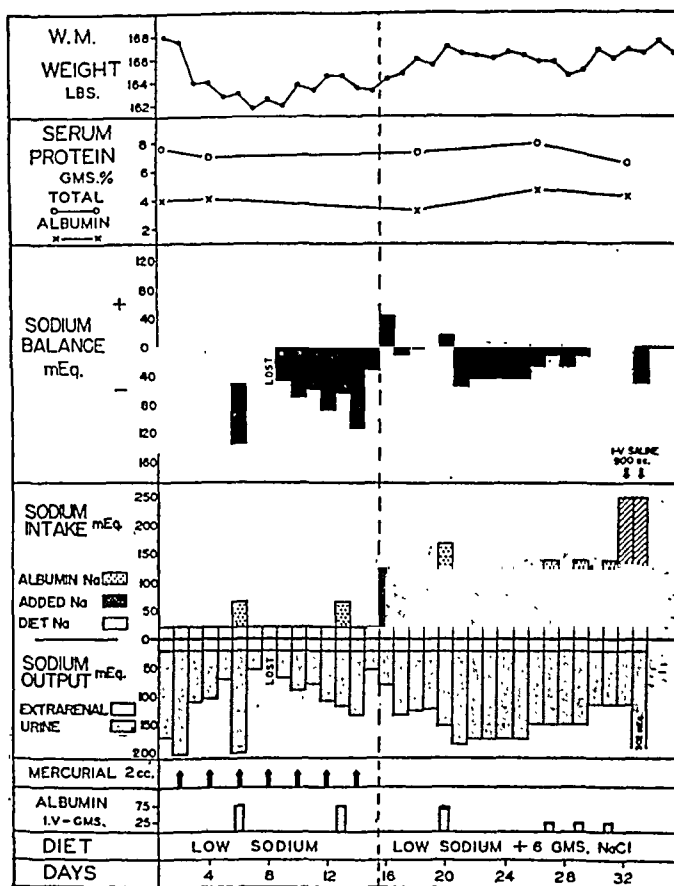


FIG. 7

days was recorded, and thereafter in spite of the added salt and further increments to his sodium intake by the administration of 900 cc. of normal saline intravenously daily for two days, weight remained constant. This patient had ceased to form ascites and, in spite of a high sodium intake, was able to excrete it apparently normally. It was previously observed that patient G. C. (Table I), while undergoing diuresis after serum albumin therapy, was likewise able quantitatively to excrete administered sodium.

DISCUSSION

The patients with cirrhosis of the liver and with demonstrable fluid retention reported here excreted in the urine but small quantities of orally or intravenously administered sodium. Positive sodium balance thus inevitably occurred. Sodium excretion increased, occasionally to permit negative sodium balance, in three circumstances: during a spontaneous decrease in ascites and edema formation; following the intravenous administration of albumin; and accompanying the parenteral exhibition of mercurial diuretics. The influence of these circumstances on fluid retention varied greatly from patient to patient. Thus, following intravenous albumin administration one patient who had never required paracentesis had a massive and prolonged diuresis with a marked increase in urinary sodium excretion. In other patients with ascites requiring repeated paracenteses the increased sodium excretion, although evident, was transitory and not great enough to lead to negative sodium balance. Likewise, the effect of mercurial diuretics on sodium excretion and diuresis was variable; one patient achieved a slow but prolonged diuresis with a considerable increase in urinary sodium excretion, while in another the effect was only transitory.

The rate of ascites formation could be altered by changing the quantity of sodium administered to patients with chronic cirrhosis in whom repeated paracenteses were necessary. Thus, a decrease in sodium intake slowed the rate of ascites formation and, conversely, fluid retention increased as more sodium was administered. This relationship held true despite the maintenance in some instances of a serum albumin concentration at or near normal by means of intravenous albumin administration. These observations place on a firmer ground the use of a low sodium diet to reduce the rate of ascites accumulation (19). Such a diet must be prepared with care so that it will furnish an ample supply of calories, protein, and vitamins, and, at the same time be palatable. The preparation and use of such a diet are published elsewhere (20).

A satisfactory explanation of the failure to excrete administered sodium is not at hand. Ralli and her co-workers (3) demonstrated an antidiuretic effect similar to that of pituitrin in the urine of patients with cirrhosis and ascites. How-

ever, retention of sodium is not one of the primary effects of the posterior pituitary antidiuretic substance (21, 22). The observations reported here suggest that sodium excretion is primarily at fault and therefore point rather to the antidiuretic effect of the sex hormones or of those elaborated by the adrenal cortex. It need not be postulated, however, that an antidiuretic is the sole or even most important cause of the failure of sodium excretion in cirrhosis. Other factors such as altered renal blood flow and glomerular filtration are being studied (16). At present too little data are available to draw definite conclusions in this regard.

The results reported here do not precisely define the relative importance in the formation of ascites, of the serum albumin concentration, the portal hypertension, or the failure to excrete administered sodium. Each is undoubtedly important, although recent studies tend to minimize the importance of the serum colloid osmotic pressure (3, 23).

SUMMARY

1. The patients with cirrhosis of the liver with ascites reported in this communication excreted only small quantities of sodium in the urine regardless of either the quantity of salt administered, or the route of its administration (oral and intravenous). The failure to excrete sodium in amounts adequate to achieve sodium equilibrium occurred in spite of the maintenance of a normal serum albumin concentration by repeated injections of concentrated salt-poor human serum albumin.
2. The urinary excretion of sodium was usually increased by the administration of mercurial diuretics or of salt-poor human serum albumin (intravenously) and also during a spontaneous decrease in ascites and edema formation.
3. A low sodium but otherwise adequate diet decreased the rate of ascites formation, while larger quantities of administered sodium led to a proportionately increased rate of formation.
4. Although several factors might have led to the failure of these patients to excrete sodium, there was no evidence that any one was solely implicated.
5. The relative importance of the failure to excrete sodium, of hypoalbuminemia, and of portal

hypertension in the genesis of ascites could not be precisely defined.

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The authors wish to express their gratitude to Alice N. Ballou, Ellen V. Cobino, Catherine M. Murphy, and Ellen Doyle for technical assistance, and to Kathleen A. Clinton for preparation of the diets used in this study.

Since submitting this paper for publication, Farnsworth and Krakusin (Farnsworth, E. B., and Krakusin, J. S., *Electrolyte partition in patients with edema of various origins. Qualitative and quantitative definition of cations and anions in hepatic cirrhosis*, *J. Lab. & Clin. Med.*, 1948, 33, 1545) have likewise demonstrated a failure in patients with cirrhosis of the liver to excrete administered sodium in the urine.

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IMPROVEMENT OF ACTIVE LIVER CIRRHOSIS IN PATIENTS MAINTAINED WITH AMINO ACIDS INTRAVENOUSLY AS THE SOURCE OF PROTEIN AND LIPOTROPIC SUBSTANCES^{1,2}

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An adequate, nutritious diet is the basic consideration in the treatment of patients with liver disease, particularly cirrhosis (1). However, because of the frequent digestive disturbances encountered in these patients, occasions arise when an adequate intake of nutritious foods cannot be maintained, and complementary or complete nutritional feeding by the intravenous route would appear indicated. Infusions of glucose and vitamins prepared for parenteral use are widely employed, but the desirability of administering protein hydrolysates intravenously to patients with hepatic insufficiency has been the subject of controversy. Thus, it has been stated that amino acids administered parenterally to such patients are not as promptly deaminized and result in a higher incidence of untoward reactions than when administered to normals (2-4), so that "intravenous injection is contraindicated in severe hepatic insufficiency" (5). Nevertheless, since other investigators have been unable to confirm any significant decrease in the rate of deamination of amino acids following their intravenous injection or any increase in untoward reactions (6-9), parenteral protein hydrolysates have been quite extensively employed as a therapeutic adjunct in the treatment of patients with cirrhosis of the liver and with infectious hepatitis (10-12).

The experiments reported here were undertaken to clarify the controversy regarding the desirability of administering protein hydrolysates pa-

renterally to patients with liver disease. Observations were made of the clinical tolerance, the metabolism and excretion, and the therapeutic value of a 10 per cent solution of amino acids administered intravenously in amounts of 500 to 1,000 cc. daily for from two to four weeks as the sole source of nitrogen and of lipotropic substances to four patients with active cirrhosis of the liver. The results indicate that intravenously administered amino acids are well tolerated by patients with severe liver disease, are metabolized and excreted in a manner differing little from normal, and may promote clinical improvement when administered as the sole source of nitrogen and of lipotropic substances.

MATERIALS AND METHODS

The clinical and laboratory observations of the four patients with cirrhosis of the liver selected for this study are tabulated in Table I. Each gave a history of longstanding alcoholism and of meager dietary intake. Three of the patients (B. B., W. C., and J. Q.) were deeply jaundiced and acutely ill at the time the study was begun.

A purified diet adequate in calories but devoid of protein (supplying only 0.1 gram of nitrogen daily as determined by macro-Kjeldahl analysis⁴) was used in all studies. Its composition and ability to maintain nitrogen balance and constant weight with the addition of 85 grams of purified casein ("Labco") as the sole source of nitrogen have previously been described (13). The diet contained no source of the vitamin B complex except choline in small amounts (30 to 100 mgm. daily as estimated from standard food tables). A capsule⁵ containing vitamins A, C, D, B₁, B₂, niacin, B₆, and pantothenic acid was given daily.

Protein was supplied intravenously as a 10 per cent solution of amino acids⁶ prepared by the recombination

⁴ One patient (J. G.) received 15 grams of "Nestle" daily, increasing the nitrogen intake by 0.25 gram.

⁵ "Dayamin" supplied through the courtesy of the Abbott Laboratories, North Chicago, Illinois.

⁶ Developed and distributed by Merck and Company, Inc., Rahway, New Jersey, who supplied the material used in this investigation.

¹ The expenses of this investigation were defrayed in part by a grant from Merck and Company, Inc., Rahway, New Jersey, to Harvard University.

² A preliminary report of this investigation was included in the Proceedings of the Fortieth Annual Meeting of the American Society for Clinical Investigation, May 3, 1948.

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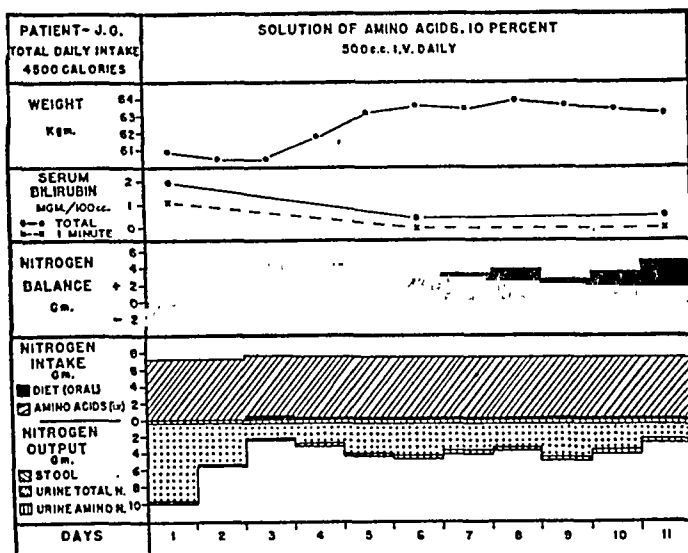


FIG. 1

of fractions of an acid hydrolysate of casein. The product contains no peptides, is essentially devoid of glutamic and aspartic acids, and is supplemented with dl-tryptophane and glycine so that it contains the eight amino acids essential for man (14). The composition of the solution employed (average of the nearly identical lots) and the quantity of amino acids infused are tabulated in Table II. The results are based on analyses by microbiological assay (15).

The basal diet was ingested at regular meal hours. The day's protein (500 to 1000 cc. of the 10 per cent solution of amino acids) was administered in one rapid intravenous injection each morning, usually over a period of one-half to one and one-half hours. Hypertonic glucose (50 to 100 grams) was added to the infusion mixture, and the injection was made one hour after a 1,000 calorie breakfast, in order to achieve maximum utilization of the infused amino acids by the simultaneous provision of adequate carbohydrate and protein (16). The total intake for the four patients during the study averaged 4,500 (J. G.), 1700 (B. B.), 3,500 (W. C.), and 4,200 (J. Q.) calories daily.

The daily urine and pooled stool nitrogen analyses were determined by the standard micro- or macro-Kjeldahl methods. The alpha amino nitrogen was determined by the gasometric ninhydrin method as described by Hamilton and Van Slyke for blood (17), and by Van Slyke, MacFadyen, and Hamilton for urine (18). The serum non-protein nitrogen was determined by Folin's micro-Kjeldahl method with direct nesslerization (19). The urines were assayed for the eight amino acids essential for man and for arginine and histidine by the microbiological method of Stokes *et al.* (15). As measurements of liver function, the serum bilirubin (20), thymol turbidity (21) and flocculation, urine urobilinogen, plasma prothrombin concentration, bromsulphalein retention, and serum albumin concentration (22, 23) were determined. Tissue obtained by punch biopsy ("Vim"-Silverman needle) from the liver of one subject both preceding and following the study period was fixed in 10 per cent

formalin, stained with hematoxylin and eosin, and examined microscopically.

RESULTS

The amino acid mixture was well tolerated clinically. No untoward reactions were noted by patients J. G. and J. Q., even though the infusion of 50 grams of amino acids was usually completed within one hour. A transient diminution of appetite was noted by patient W. C. following several of the infusions, but rarely persisted for more

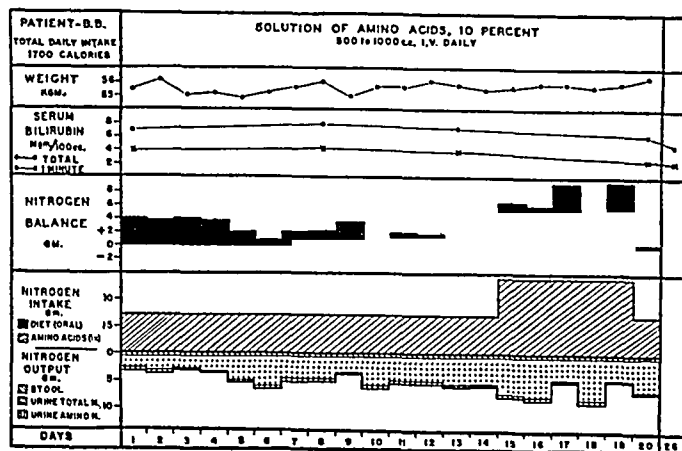


FIG. 2

than one or two hours. Nausea and retching occurred on three occasions following an attempt at forced feeding of sweetened drinks immediately after the infusion. Patient B. B. complained of constant anorexia that both preceded and followed the present study. Although this digestive disturbance was not aggravated further by infusions of 500 cc. of the amino acid solution, 1,000 cc. infusions, even though administered more slowly, were followed on three occasions by nausea and vomiting. Thrombosis of the infused vein did not occur in any patient. An increased incidence of untoward reactions was not observed while the patients were deeply jaundiced and acutely ill. Despite the severity of the patients' illness and the rapidity of the infusions, distressing symptoms were noted but six times following 79 infusions—a reaction incidence of less than 8 per cent.

The nitrogen balance data for these patients is summarized in Table III and plotted in Figures 1-4. Patient J. G. maintained a positive nitrogen balance throughout the 11 day study period, except for day one. Although the daily infusion of

50 grams of amino acids (500 cc. of the 10 per cent solution) to this subject, together with the diet, supplied slightly less than 0.8 gram of protein per kilogram of body weight, an average positive balance of 3.0 grams of nitrogen daily was realized. Patient W. C. received 100 grams of amino acids (1,000 cc. of the 10 per cent solution) intravenously daily for 18 days and retained a total of 50.8 grams of nitrogen, or an average positive balance of 2.8 grams of nitrogen daily. During a 15 day period while patient B. B. received 50 grams of amino acids parenterally daily, the nitrogen balance averaged +2.2 grams daily, and increased to +7.4 grams of nitrogen daily for five days when the quantity of protein administered was doubled. The excretion of nitrogen in the urine increased little despite the large increase in the quantity of amino acids infused. Patient J. Q. was in slight positive nitrogen balance only during the final six days of a 30 day study period while receiving 100 grams of amino acids intravenously daily. Nitrogen balance was persistently

Thus the negative nitrogen balance persisted unaltered until 100 grams of the amino acid mixture was supplied each day.

The serum concentration of alpha amino and of non-protein nitrogen and the urinary excretion of alpha amino nitrogen in each individual did not vary greatly during the study (Table IV). Since the amino acid content of serum is usually 10 to 40 per cent greater than that of plasma (24), the normal range of alpha amino nitrogen in this laboratory of from 2.9 to 4.6 mgm. per cent for plasma (9) would be increased to from 3.2 to 6.4 mgm. per cent for serum. Although several of the fasting serum alpha amino nitrogen values obtained in the patient (W. C.) receiving 100 grams of amino acids intravenously daily for 18 days were elevated, the values averaged 5.9 mgm. per 100 cc. of serum which is within the normal range.

A slight weight gain occurred in each patient (Figures 1-4). Minimal peripheral edema developed in patient J. G. during the first five days of the study coincident with the inadvertent provision of supplemental salts (5 grams NaCl and 1 gram KCl), and subsided with their cessation. Although sodium, other than that present in the basal diet, was not supplied the other patients, definite peripheral edema occurred in patient J. Q. and increased in degree as the gain in weight continued, and was associated with a fall in the plasma albumin concentration from 2.6 to 1.8 grams per 100 cc. Edema was not discernible in the other patients.

Improvement in liver function tests was observed in all patients during the period of study

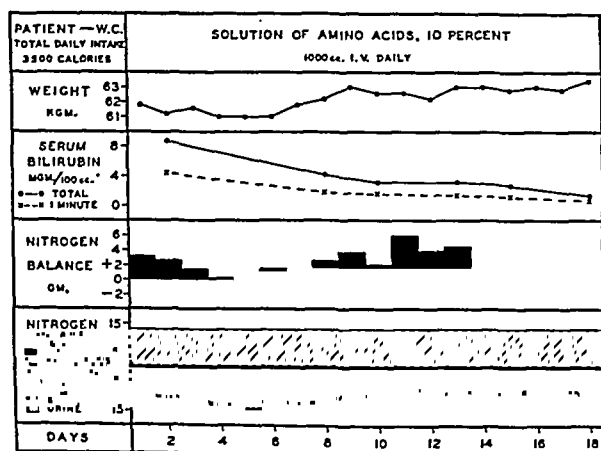


FIG. 3

negative throughout a preceding 22 day period while receiving 50 grams of amino acids intravenously daily and a two day period while receiving 75 grams daily. During the protracted period of negative nitrogen balance, the nitrogen-sparing effects of individual amino acid supplements were investigated. The addition of dl-phenylalanine (2 grams) and of dl-threonine (3 grams) individually and combined, given by mouth one hour prior to the intravenous infusion, had no apparent effect on the quantity of nitrogen excreted in the urine.

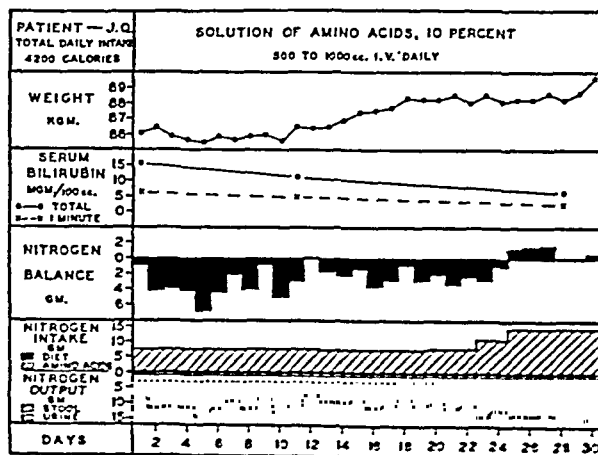


FIG. 4

TABLE I
Clinical and laboratory observations of patients with cirrhosis of the liver

Patient (Age, Sex)	J. G. (31, Male)		B. B. (45, Female)		W. C. (53, Male)		J. Q. (48, Male)		
Condition of study	Before study	After study	Before study	After study	Before study	After study	Before study	After study	
Jaundice*	0	0	3+	2-3+	3+	0	4+	2+	
Spider angiomata*	2+	2+	4+	4+	0	0	2+	2+	
Liver size (cm.)	4	4	16	16	2	0	11	7	
Spleen size (cm.)	0	0	2	0	0	0	3	1	
Ascites*	0	0	2-3+	2+	±	0	1+	1+	
Peripheral edema*	0	1+	0	0	0	0	0	1+	
Serum bilirubin (mgm. per 100 cc.)	1 min.	1.1	0.1	3.8	2.6	4.5	0.8	6.6	2.6
	Total	1.9	0.7	7.3	6.4	8.7	1.2	15.5	6.6
Thymol turbidity† (cc. BaSO ₄)	0.7	0.6	2.8	2.4	3.9	2.7	4.5	3.2	
Thymol flocculation*	0	0	3+	1+	3+	1+	1+	1+	
Urine bile*	0	0	2+	1-2+	3+	0	4+	2+	
Urine urobilinogen			1:256		1:256		1:80		
Prothrombin time (per cent of normal)	73		43		60		35		
Serum proteins (grams per 100 cc.)	Total	6.5	6.0	5.8	5.8	5.7	6.7	5.2	
	Albumin	4.1	2.1	2.4	3.1	2.2	2.6	1.8	
Bromsulphalein retention (per cent)‡		14				20			

* 0 to 4+.

† Normal range: Below 1.7 cc. BaSO₄.

‡ At 45 minutes after intravenous injection of 5 mgm. dye per kilogram of body weight.

(Table I, Figures 1-4). A progressive decline in the serum bilirubin, degree of jaundice, and urine bile was the most marked change noted. The thymol turbidity and thymol flocculation tests were found abnormal in three patients before the study, and in each instance showed slight change toward normal. In three instances where the serum protein concentrations were determined before and after the study period, the total protein remained relatively constant, while the albumin rose slightly in one and fell in the others. By physical examination, in addition to a lessening of jaundice, it was noted that the liver size decreased in two patients, and remained unchanged in the others. The spleen became less readily palpable in the two patients with splenomegaly. The degree of ascites, when present, was not appreciably altered.

As mentioned previously, peripheral edema became evident in two patients. In addition to generally improved liver function tests and physical findings, each patient experienced subjective improvement. This was evidenced by increased physical and mental activity, a greater desire to eat, and an enhanced sense of well-being.

Liver punch biopsies obtained from patient W. C. prior to the study and at its conclusion (20 day interval) were reported as follows:⁷

An adequate portion of liver was obtained. The

⁷ The authors gratefully acknowledge the assistance of Norman Zamcheck, M.D., in the pathological interpretation of these specimens. It is regretted that liver biopsy was contraindicated in patients B. B. and J. Q. because of a prolonged prothrombin time (not responding to vitamin K therapy) and deep jaundice.

TABLE II

Composition of the 10 per cent solution of amino acids

	Amounts of amino acids in 500 cc. of the 10 per cent solution*
	grams
Arginine	1.3
Histidine	2.0
Isoleucine	4.2
Leucine	8.4
Lysine	5.2
Methionine	2.0
Phenylalanine	2.1
Threonine	0.9
Tryptophane	0.45
Valine	3.5

* Determinations by microbiological assay (1-form) (15). Also contains: 0.45 gram d-tryptophane, 0.1-0.3 gram glutamic acid, 0.1 gram tyrosine, 11.3 grams glycine, less than 0.03 gram aspartic acid, and (by difference) 3.5 grams non-assayed amino acids per 500 cc. of the 10 per cent solution. Total 10 "essential" amino acids comprise 64 per cent of total amino acids in mixture. Each 500 cc. infusion (by analysis) supplies 44 grams of amino acids, 7.0 grams of total nitrogen, and 6.0 grams of alpha amino nitrogen.

appearance was consistent with portal cirrhosis. Marked fibrosis of the portal areas was noted throughout. A moderate number of lymphocytes and a few eosinophiles were scattered through the fibrous tissue. Bile duct proliferation was prominent. A small amount of fat was scattered in focal areas through the liver parenchyma and the liver cells contained only minimal amounts of glycogen. "Alcoholic hyaline" was found. Many bile thrombi were seen obstructing and distending the bile canaliculi.

The second biopsy obtained 20 days later showed

distinct improvement in the histologic appearance of the liver structure as follows:

Most notable was a marked increase in cell content of glycogen which distended the liver cells so that they appeared to be increased in size and obscured adjoining sinusoids in many areas. There was a decrease in fat although this had not completely disappeared. No bile thrombi were seen. A few cells contained a small amount of green staining pigment and more green pigment was collected in scattered Küpfer cells. Less striking changes consisted of an apparent decrease both in amount of fibrous tissue and of cellular infiltrate of the portal areas.

The loss in the urine of alpha amino nitrogen and of each of the 10 "essential" amino acids following the infusion of the amino acid solution is tabulated in Table V.⁸ There was a loss from ex-

⁸ A portion of the amino acids excreted into the urine is of endogenous source (25). All values listed in Table V are in excess of the amount of alpha amino nitrogen or of individual amino acids excreted by patient W. C. the second day while receiving a 3,500 calorie, protein-free diet prior to the present study. These "endogenous" values were: arginine 8.3; histidine 71.0; isoleucine 5.3; leucine 12.6; lysine 17.4; methionine 4.1; phenylalanine 14.8; threonine 19.1; tryptophane 6.9; valine 7.6; and alpha amino nitrogen 113 mgm. per 24 hours. Although it would have been desirable to obtain "endogenous" amino acid excretion values for each patient, it was not deemed advisable to withhold protein from their diets, particularly since the values for patient W. C. agreed quite closely with those found in the normal (25). Actually, the "endogenous" values are so small in relation to the quantity of amino acids lost following infusions of protein hydrolysates as to be relatively insignificant.

TABLE III

Nitrogen balance data of four patients with cirrhosis of the liver given 500 to 1,000 cc. of a 10 per cent solution of amino acids intravenously daily

Patient	J. G.	B. B.		W. C.	J. Q.			
Duration of study	11 days	15 days	5 days	18 days	22 days	2 days	6 days	
Amounts given daily	50 grams	50 grams	100 grams	100 grams	50 grams	75 grams	100 grams	
Nitrogen intake } grams	Intravenous	78.3	106.8	71.0	234.9	166.5	22.1	88.2
	Diet	4.0	0.8	0.3	1.6	2.3	0.2	0.6
	Total	82.3	107.6	71.3	236.5	168.8	22.3	88.8
Nitrogen output } grams	Urine	44.6	67.0	31.9	180.1	208.2	24.0	78.2
	Stool	4.4	7.5	2.5	5.6	22.4	2.0	6.1
	Total	49.0	74.5	34.4	185.7	230.6	26.0	84.3
Nitrogen balance } Total		+33.3	+33.1	+36.9	+50.8	-61.8	-3.7	+4.5
	Av./day	+3.0	+2.2	+7.4	+2.8	-2.8	-1.9	+0.8

well as of alpha amino nitrogen, excreted into the urine (Table V). However, it is apparent that certain of the amino acids were more freely lost than were others. Thus, as a group, the patients excreted fairly large percentages of administered threonine and histidine, a small percentage of arginine, and intermediate percentages of the other amino acids. The percentage of individual amino acids and of alpha amino nitrogen given which was lost in the urine by patient J. Q. was as great when 50 grams were given daily and negative nitrogen balance resulted, as when he received twice the quantity and nitrogen equilibrium was attained.

The patterns of the 10 "essential" amino acids in the solution administered intravenously and in the urine are shown in Table VI and plotted in Figure 5. The proportion of the "essential" amino acids in the mixture differed considerably from that excreted in the urine. For example, although arginine and histidine were present in similar proportions in the mixture infused (4.2 and 6.6 per cent of the total 10 "essential" amino acids, respectively), their pattern in the urine was altered so that proportionately far more of the 10 amino acids excreted (average of the four patients) was composed of histidine (15.6 per cent) than of arginine (0.7 per cent). The amino acid pattern

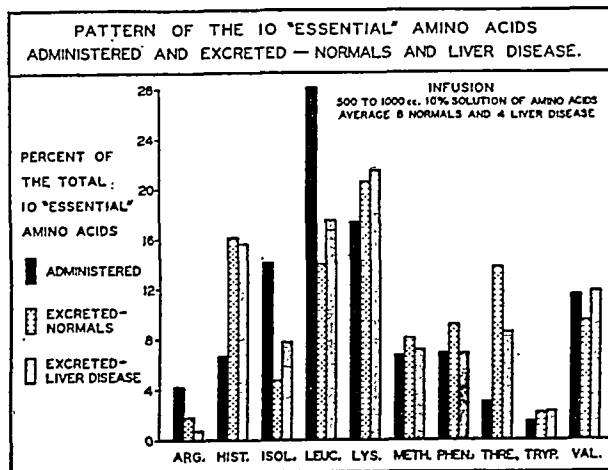


FIG. 5

in the urine following the larger (100 gram) infusions in patients W. C. and J. Q. more closely resembled the pattern of the solution given than that following the 50 gram infusions in patients J. G. and B. B.

DISCUSSION

Patients with cirrhosis of the liver can maintain a positive nitrogen balance if adequate dietary protein is given (26, 27). When an adequate dietary intake of protein can not be maintained, it would appear desirable to provide the protein in a hydrolyzed form intravenously. None of the objections raised to the administration of protein hydrolysates intravenously to patients with liver disease has gone unchallenged, and the present paper suggests that they were unfounded.⁹

I. Clinical tolerance of parenterally administered amino acids in liver disease

For several years in this clinic protein hydrolysates have been administered intravenously as a therapeutic adjunct in the treatment of patients with liver disease. The protein hydrolysates employed (both acid and enzymatically hydrolyzed) were as well tolerated clinically by patients with hepatitis (infectious, toxic, homologous serum),

⁹ Because of improved methods of manufacturing protein hydrolysates, many of the untoward reactions of a decade ago are rarely encountered today. Impressions and conclusions made when less refined products were employed in the past should not necessarily continue to serve as criteria of the value (or lack of value) of hydrolysates now used.

TABLE VI

Comparison of the patterns of the 10 "essential" amino acids administered and excreted following amino acid infusions to patients with liver disease and normal subjects

	Per cent of total of 10 "essential" amino acids						
	Given*	Excreted in liver disease†					
		J. G.	B. B.	W. C.	J. Q.	Average	Excreted in normals‡
Arginine	4.2	0.5	1.0	0.7	0.4	0.7	1.8
Histidine	6.6	21.5	20.0	7.5	13.2	15.6	16.1
Isoleucine	14.1	5.8	5.9	10.9	8.6	7.8	4.7
Leucine	28.1	9.2	14.9	22.3	23.5	17.5	14.0
Lysine	17.3	33.2	21.0	17.3	14.7	21.5	20.6
Methionine	6.7	5.2	5.4	7.4	10.9	7.2	8.1
Phenylalanine	6.9	4.1	6.8	8.5	8.2	6.9	9.2
Threonine	3.0	10.4	9.8	7.6	6.7	8.6	13.8
Tryptophane	1.5	2.3	2.6	2.0	2.3	2.3	2.2
Valine	11.6	7.8	12.6	15.8	11.5	11.9	9.5

* Calculated from the quantity of amino acids given, Table II.

† Calculated from the quantity of amino acids excreted, Table V. Excretion pattern of patient J. Q. calculated from 100 grams period.

‡ Average value of eight infusions in normal subjects given 500 cc. of the 10 per cent solution of amino acids intravenously (35).

cirrhosis of the liver (Laennec's, biliary, toxic, pigment), Wilson's disease, liver coma, and obstructive jaundice, as they were by patients with other illnesses or by normal individuals. The relative freedom from untoward reactions following the rapid and repeated daily infusions for from two to four weeks of from 500 cc. to 1,000 cc. of the 10 per cent solution of amino acids to the four patients with severe liver disease observed in the present study confirms the previous impression that amino acids may be administered intravenously with relative impunity to patients with liver disease. The reaction incidence (nausea) of less than 8 per cent noted in the patients with liver disease was similar to that observed in a larger series of individuals following infusions of the same hydrolysate (28).

II. Metabolism of parenterally administered amino acids in liver disease

Evidence that the intravenously administered amino acids were normally metabolized by the patients with liver disease was the positive nitrogen balance attained. Thus, three of the patients (J. G., B. B., and W. C.) maintained a positive nitrogen balance with an average retention of 3 grams of nitrogen daily throughout the period of study. The nitrogen balance in one of these patients increased from +2.2 grams daily to +7.4 grams daily when the quantity of protein administered was doubled. The fourth patient (J. Q.) was in slight positive nitrogen balance while receiving 100 grams of amino acids daily for six days. The latter individual, however, failed to maintain nitrogen equilibrium while receiving 50 and 75 grams of amino acids intravenously daily for 22 and for two days, respectively. Oral supplements of phenylalanine and threonine, amino acids which favor nitrogen conservation in dogs (29), did not alter the negative nitrogen balance. The provision of 50 grams of amino acids to this patient of large stature only represented approximately 0.5 gram of protein per kilogram of body weight which borders on the minimum oral requirement (30). Furthermore, the quantitative requirement of hydrolyzed protein administered intravenously is likely greater than that of an equivalent quantity of whole or of hydrolyzed protein given orally (31, 32). Because the latter patient maintained

nitrogen equilibrium while receiving 100 grams of amino acids intravenously daily, it is suggested that during the protracted period of negative nitrogen balance he received an insufficient quantity of amino acids to fulfill the body protein requirements, particularly in view of the adequate caloric intake maintained throughout the study (4,200 calories daily). Thus, the provision of adequate quantities of amino acids intravenously permitted each of the patients with cirrhosis of the liver to achieve a positive nitrogen balance. The positive nitrogen balance did not reflect a retention of nitrogenous products in the blood, for the serum non-protein nitrogen remained constant throughout the study (Table IV).

Further evidence that the infused amino acids were completely metabolized was the failure to observe a progressive rise in the serum alpha amino nitrogen concentration in the patient receiving 100 grams of amino acids intravenously daily for 18 days (W. C., Table IV). Although the ability of an impaired liver to deaminate promptly intravenously administered amino acids has been questioned, the blood amino acid values following infusions of protein hydrolysates do not differ greatly in normals and in patients with liver disease, as shown in Figure 6. Thus, patients with liver disease are able to metabolize intravenously injected amino acids promptly and completely, whether given once as in the "tolerance tests" reported in the literature (2, 7-9) and plotted in Figure 6, or given repeatedly as in the present study.

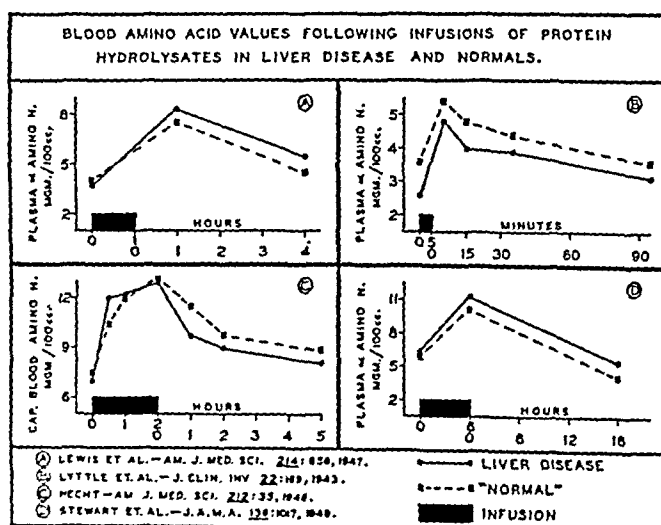


FIG. 6

The significance of the slight weight gain observed in each of the patients (Figures 1-4) is difficult to evaluate. Gain in weight was associated entirely in one patient (J. Q.) and, at least in part, in a second (J. G.) with fluid retention, since both developed minimal peripheral edema during the study. Since patients with "decompensated" cirrhosis of the liver have an avidity for sodium and water (33), it can not be stated with certainty that fluid retention did not occur in all of the patients in this study.

III. *Excretion of parenterally administered amino acids in liver disease*

It was reported previously from this clinic that the average daily excretion of alpha amino nitrogen and of the 10 "essential" amino acids in the urine of patients with severe liver disease eating *ad libitum* did not differ markedly from normal subjects, although there was considerable variation among patients in the quantity of individual amino acids excreted (34). Similarly, the "endogenous" excretion of amino acids by patient W. C.^a was similar to that found in the normal (25). The present study presents further evidence that parenterally administered amino acids are excreted in patients with severe liver disease in a manner differing little from normal. The loss of alpha amino nitrogen in the urine by the patients with liver disease (Table V) was similar to that observed in a previous study in normals following infusions of equivalent quantities of the same hydrolysate (35). Thus, there was an average urinary loss following 500 cc. infusions of 9 per cent (range 3 to 13 per cent) of the amino nitrogen administered to normal subjects and of 3 and 6 per cent to patients with liver disease (J. G. and B. B.). The urinary loss of amino nitrogen following 100 gram amino acid infusions averaged 14 per cent (range 9 to 19 per cent) in normals and 4, 14, and 20 per cent in patients with liver disease (B. B., W. C., and J. Q.). Furthermore, as in the normal individuals, the urinary excretion loss of alpha amino nitrogen of the patients with liver disease correlated more closely with the size of the infusion than with the speed at which it was given. A progressive increase in the urinary excretion of alpha amino nitrogen did not occur in any patient during the study (Table IV).

The observation that patients with severe liver disease excreted fairly large percentages of administered threonine and histidine and smaller percentages of the other amino acids following infusions of the amino acid solution is further evidence that amino acid excretion in liver disease is normal, since these amino acids were similarly excreted following amino acid infusions in normal subjects (35). Furthermore, the post-infusion pattern of the "essential" amino acids excreted in the urine of the patients with cirrhosis was similar to that in normals, although the individual amino acids were not excreted in the same proportion as administered in either group (Table VI, Figure 5). The excretion pattern following the larger (100 gram) infusions in the patients with liver disease more closely resembled the pattern of the solution given than that following the 50 gram infusions. It was previously observed in normals that the amino acid pattern following rapid infusions of the amino acid solution more closely resembled the pattern of the amino acids given than that following infusions given more slowly (35). Thus, the administration of amino acids intravenously slowly and in small quantity is followed by excretion in the urine of but minimal amounts of amino acids in a pattern differing from the pattern of the amino acids given, while the infusion of amino acids rapidly and in large amounts results in a greater excretion loss of alpha amino nitrogen and of individual amino acids in a proportion quite similar to that in the solution administered. As discussed previously (35), an interpretation of these observations is not possible until further data relating to renal clearance studies in man are available. Nevertheless, the similarity in the renal excretion of amino acids by normals and by patients with liver disease is apparent.

IV. *Therapeutic value of parenterally administered amino acids in liver disease*

Improvement in the liver disease was observed in all patients during the period of study, as evidenced by a progressive decline in the serum bilirubin, degree of jaundice, and urine bile in each patient, a decrease in the size of the liver and spleen in two patients, and a distinct improvement in the histologic appearance of the liver structure in the second biopsy obtained in one patient. The im-

provement in the liver function tests and physical findings was as marked when insufficient amino acids were administered intravenously and negative nitrogen balance resulted (patient J. Q., Table I and Figure 4), as when sufficient amino acids were available to attain positive nitrogen balance. This finding suggests that improvement in liver function in man can proceed even in the face of a negative nitrogen balance, perhaps as a result of mobilization of tissue proteins. That repair of injured tissue can take place during periods of insufficient dietary protein intake and negative nitrogen balance by the diversion of nitrogen from one organ of the body to another finds support in several studies. Thus, some replacement of liver protein occurs in partially hepatectomized rats maintained on a protein-free diet (36), blood regeneration proceeds satisfactorily in anemic animals and man during periods of negative nitrogen balance (37, 38), and considerable tissue repair (fibroplasia) occurs in the experimental wounds of rats maintained on a low protein diet (39) and presumably also during the "catabolic period" after damage in man. Under these circumstances, however, an adequate protein intake and retention would be necessary in order to replace the depleted tissue protein, and perhaps to enhance repair of the injured tissue.

Further studies in man are needed to determine whether liver repair is more satisfactorily achieved by an adequate rather than by an inadequate protein intake, or by diets containing supplements of additional protein or of "lipotropic" substances. The present study does demonstrate, however, that liver repair in man can proceed when amino acids, even in amounts insufficient for nitrogen equilibrium, are administered intravenously as the sole source of nitrogen and of lipotropic substances except for small amounts of choline.

Post and Patek observed that patients with cirrhosis of the liver absorb and retain food protein as do patients with simple protein starvation, and differ from the latter group only by an impaired ability to synthesize serum albumin (27). The difficulty of increasing the serum albumin concentration in undernourished patients with chronic liver cirrhosis by feeding a nutritious diet high in protein is well established (40, 41). Thus, it is not surprising that the serum concentration of total

protein and of albumin did not rise in the patients during the present study (Table I).

It should be emphasized that the present study was undertaken to determine the clinical tolerance, metabolism and excretion, and therapeutic value of amino acids administered intravenously to patients with liver disease. It is neither the authors' intent nor desire to suggest that patients with liver disease receive each day a synthetic protein-free diet by mouth, and 50 to 100 grams of amino acids by vein as the sole source of nitrogen and of lipotropic substances. We firmly believe that therapy in liver disease is based upon the fundamental principle of insuring that nutritious food is eaten, digested, and absorbed. When an adequate intake of nutritious foods can not be maintained, however, the results of the present study indicate the desirability of providing the protein nutrients intravenously as amino acids.

SUMMARY

1. Four patients with active cirrhosis of the liver were given a protein hydrolysate intravenously daily for from two to four weeks as the sole source of nitrogen. A purified diet furnished adequate calories, but only small amounts of choline.

2. The amino acid solution (50 to 100 grams of amino acids) was administered in one rapid injection each morning and was well tolerated clinically.

3. The provision of adequate quantities of amino acids intravenously permitted each of the patients to achieve a positive nitrogen balance which was not associated with azotemia or hyperaminoacidemia.

4. There was a loss into the urine of from 3 to 23 per cent of the alpha amino nitrogen administered. This loss correlated more closely with the size of the infusion than with the speed at which it was given, and did not progressively increase in any patient during the study. The individual amino acids were not excreted in the urine in the same proportion as administered, so that as a group the patients excreted fairly large percentages of administered threonine and histidine, a small percentage of arginine, and intermediate percentages of the other amino acids. These data are similar to those observed in normals.

5. Improvement in the liver disease was observed in all the patients during the period of study, as evidenced by a progressive decline in the serum bilirubin, degree of jaundice, and urine bile in each patient, a decrease in the size of the liver and spleen in two patients, and a distinct improvement in the histologic appearance of the liver structure in one patient.

6. Improvement in liver function tests and physical findings was even observed in one patient who received insufficient amino acids and was in negative nitrogen balance for three weeks. This finding suggests that improvement in liver function in man can proceed even in the face of a negative nitrogen balance, perhaps as a result of mobilization of tissue proteins.

7. It is concluded that intravenously administered amino acids were well tolerated by these patients with severe liver disease, were metabolized and excreted in a manner differing little from normal, and permitted clinical improvement although administered as the sole source of nitrogen and of lipotropic substances.

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THE EFFECTS OF DIHYDROERGOCORNINE ON THE CIRCULATION IN THE EXTREMITIES OF MAN

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Since the advent of therapeutic surgical procedures on the autonomic nervous system, increased interest has been shown in drugs which block this fairly complex and enigmatic part of the nervous system. One of these drugs is a derivative of ergot, called dihydroergocornine (DHO-180). Some of its clinical applications have been reported by several workers, (1-6), showing principally its effects in hypertension, in which a lowering of blood pressure was obtained. Bluntschli and Goetz (2, 5) reported cases in which they recorded toe and finger volume, pulse volume and rate, and skin temperatures before and after administration of dihydroergocornine. They stated that vasodilatation and reduction of blood pressure in both normal and hypertensive subjects were produced through functional blocking of sympathetic impulses by the drug.

Ergot of rye has long been separated into component parts and many substances have been obtained from the crude drug. Stoll and his co-workers (7-9) have shown that ergot contains three alkaloidal groups, and six natural alkaloids, each of which has two isomeric forms. The three groups, and the alkaloids contained therein, are the ergotamine group, containing ergotamine and ergosine; the ergotoxine group, made up of three alkaloids, ergocristine, ergokryptine and ergocornine; and the third group with only one alkaloid, ergobasine. These alkaloids have similar chemical structures and can be transformed into dihydrogenated forms. The latter have been found to be less toxic than the natural alkaloids.

Since dihydroergocornine has been reported to have vasodilator properties by virtue of its purely sympathicolytic action (2, 3, 5, 6), we deemed it interesting to investigate its effects on the peripheral circulatory system in man, under carefully controlled laboratory conditions.

METHODS

A group of 20 patients with various primary diagnoses, including Ménière's syndrome, multiple sclerosis, headaches of various types and hypertension, volunteered for this study. The ages varied between 20 and 63 years.

Before the observations were begun, the patient reclined quietly on a comfortable test bed for at least 30 minutes, in a constant temperature room at 77° F. with a humidity of about 40 per cent. The blood flow in all four extremities was measured by means of the venous occlusion plethysmograph with a compensating spirometer recorder (10). The arm plethysmograph included the hand, wrist, forearm and part of the arm up to 2 inches (about 5 cm.) above the olecranon process. The leg plethysmograph included the foot, ankle and leg up to 1 inch (about 2.5 cm.) below the tibial tuberosity. Finger and toe pulses were recorded by means of digital plethysmographs on the right and left index fingers and on the right and left second toes.

The heart rate and the systolic and diastolic blood pressures were recorded by means of the usual clinical procedures. Control values were established for heart rate, blood pressure and blood flow before the drug was given. These observations were again repeated at regular intervals during a period averaging 65 minutes and ranging from 15 to 85 minutes after the drug had been administered. However, when the drug was given by infusion over a period of 20 minutes, additional observations were taken during the period of infusion.

Dihydroergocornine was given in total doses from 0.25 mg. to 0.4 mg. The dose varied from 0.0038 mg. to 0.008 mg. per kilogram of body weight. In six cases the drug was given by continuous intravenous infusion in physiologic saline solution containing 0.5 mg. dihydroergocornine (DHO-180) per 100 cc. and was administered over a period of 20 minutes. In 14 other cases the drug was given in a single dose by intravenous injection.

RESULTS

Table I presents the changes in blood flow in the upper and in the lower extremities as well as the changes in heart rate and blood pressure of subjects who were given dihydroergocornine by intravenous infusion in the amounts specified. The average increase in blood flow was 117 per cent for both upper extremities with a range of 35 to

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TABLE I

Change in peripheral blood flow, heart rate and blood pressure after dihydroergocornine (DHO-180) had been given by infusion

Case	Total dose	Blood flow, cc. per min. per 100 cc. of tissue Arms			Blood flow, cc. per min. per 100 cc. of tissue Legs			Heart rate, maximal change	Blood pressure, maximal change	
		Control before in- jection	Maximal change after in- jection	Per cent change	Control before in- jection	Maximal change after in- jection	Per cent change		Systolic	Diastolic
	mg.							beats per min.	mm. Hg	mm. Hg
1	0.3	3.8	10.0	+163	2.3	3.8	+ 65	- 2	- 5	0
2	0.35	6.4	12.5	+ 95	2.2	3.0	+ 36	- 2	-20	-4
3	0.3	3.2	7.5	+134	2.6	3.3	+ 27	- 6	-20	-4
4	0.4	4.3	5.8	+ 35	3.6	8.6	+139	-12	+28	+4
5	0.35	4.4	13.8	+214	2.6	6.3	+142	-14	-12	-6
6	0.3	4.9	8.0	+ 63	2.6	4.1	+ 58	-12		
Average				+117			+ 78	- 8	- 6	-2

214 per cent. The average increase in both lower extremities was 78 per cent with a range of 27 to 142 per cent. The maximal change in blood flow occurred at an average time of 43 minutes after the infusion had begun and 23 minutes after the entire amount of the drug had been given.

Table II shows the changes in blood flow in the four extremities, as well as the changes in heart

rate and blood pressure of subjects given the drug by single intravenous injection. An increase of blood flow was observed in all but one patient. The average increase was 84 per cent with a range of 25 to 170 per cent in both upper extremities. The average increase in both lower extremities was 63 per cent with a range of 8 to 112 per cent. One patient showed a decrease of 3 per cent in the

TABLE II

Change in peripheral blood flow, heart rate and blood pressure after dihydroergocornine (DHO-180) had been given by single intravenous injection

Case	Total dose	Blood flow, cc. per min. per 100 cc. of tissue Arms			Blood flow, cc. per min. per 100 cc. of tissue Legs			Heart rate, maximal change	Blood pressure, maximal change	
		Control before in- jection	Maximal change after in- jection	Per cent change	Control before in- jection	Maximal change after in- jection	Per cent change		Systolic	Diastolic
	mg.							beats per min.	mm. Hg	mm. Hg
7	0.35	8.3	11.8	+ 42	3.1	5.3	+ 71	-16	- 2	- 4
8*	0.3	3.0	8.1	+170	2.4	3.9	+ 63	-10	-30	-10
9	0.35	5.3	9.1	+ 72	5.6	8.8	+ 57	-28	-18	-14
10	0.35	5.9	11.7	+ 98	4.5	8.6	+ 91	- 8	+14	+ 6
11	0.25	2.4	5.1	+113	2.4	4.3	+ 79	-32	-16	- 4
12	0.3	9.1	11.4	+ 25	5.9	6.4	+ 8	-10	- 4	+ 4
13	0.35	3.2	4.8	+ 50	1.9	3.4	+ 79	-11	- 6	- 2
14	0.25	6.2	6.0	- 3	3.0	3.4	+ 13	-14	+ 6	+10
15	0.35	5.1	11.4	+124	2.4	4.4	+ 83	-13	-16	0
16	0.3	5.4	10.8	+100	2.5	5.3	+112	-16	-12	+ 2
17	0.3	4.9	6.4	+ 31	2.9	3.5	+ 21	- 2	- 8	-12
18	0.3	3.3	8.3	+152	2.4	3.2	+ 33	-12	- 6	0
19*	0.35	8.7	14.6	+ 68	4.3	8.2	+ 91	-24	-58	-18
20	0.3	4.5	10.4	+131	3.8	7.1	+ 87	-16	- 6	- 8
Average				+ 84			+ 63	-15	- 6†	- 2†

* Hypertensive cases.

† Exclusive of cases 8 and 19.

blood flow of the upper extremities. The overall average increase in blood flow for the entire group of 20 cases was 94 per cent in the upper extremities and 68 per cent in the lower extremities.

At an average time of 65 minutes after the drug had been given, final readings still showed a definite increase in blood flow in all four extremities.

There was no significant change in the systolic or diastolic blood pressure readings in any of the 17 normotensive patients. However, in the two hypertensive subjects (cases numbered 8 and 19 in the tables) there was a significant and marked fall in the blood pressure of both subjects. The maximal change occurred between 30 and 45 minutes after the drug had been given. Tables I and II give the changes of blood pressure on the individual cases.

The heart rate decreased in every case after dihydroergocornine was administered. The average decrease was 13 beats per minute, with a range of 2 to 32 beats per minute. Tables I and II give the changes in heart rate in the individual cases.

No toxic or cumulative effects were noted in the three patients who were given the drug from eight to 20 times once daily on successive days by infusion of 0.5 mg. of dihydroergocornine (DHO-180) in 100 cc. of physiologic saline solution.

Side reactions were common and were noted in 19 of the 20 cases, but none were serious. Seventeen patients complained of nasal congestion, which in most cases produced severe obstruction of the nasal passages. This occurred within 25 minutes after dihydroergocornine had been given and usually lasted for about two hours. Benadryl, given to a few subjects, had no effect on reducing this congestion. Mild to moderate nausea occurred in seven cases and vomiting in only one case. Headache occurred in six cases and was moderately severe. A sensation of flushing occurred for a few minutes in four cases. Four patients experienced a sudden urge to urinate. This occurred at the peak of action of the drug and was not observed in other cases during equally long and similar periods when no drug was given. No flushing of the exposed skin surface nor sweating were noted in any of the cases. No pupillary changes occurred.

Figures 1 and 2 show typical blood flow curves before and at the height of action of the drug. Figure 3 shows the increase over the control in

amplitude of the pulse in the fingers and toes after the administration of dihydroergocornine. The pulse tracings of the right and left index fingers and of the right and left second toes of 17

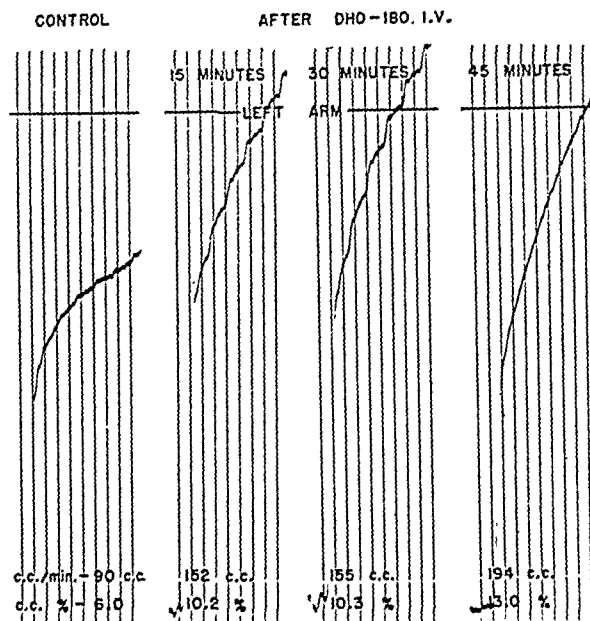


FIG. 1, a

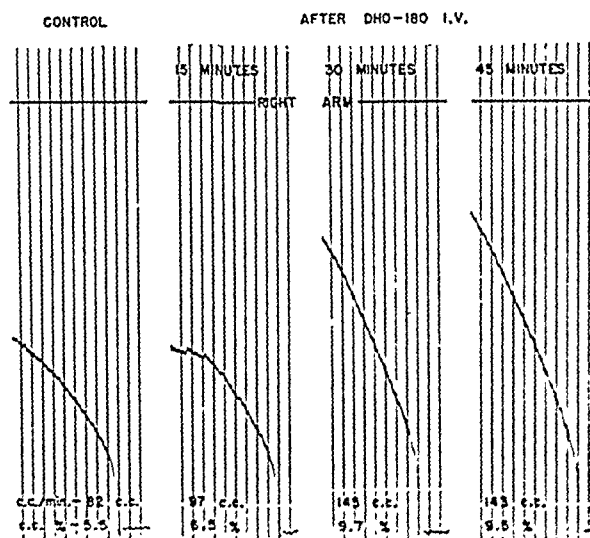


FIG. 1, b

FIG. 1, a and b. REPRESENTATIVE BLOOD FLOW CURVES BEFORE, AND 15, 30 AND 45 MINUTES AFTER THE INTRAVENOUS ADMINISTRATION OF DIHYDROERGOCORNINE

a, left arm; b, right arm.

The curves for the right arm are mirror images of those for the left because each spirometer, as a unit in the compensating mechanism, deflects the beam in the opposite direction.

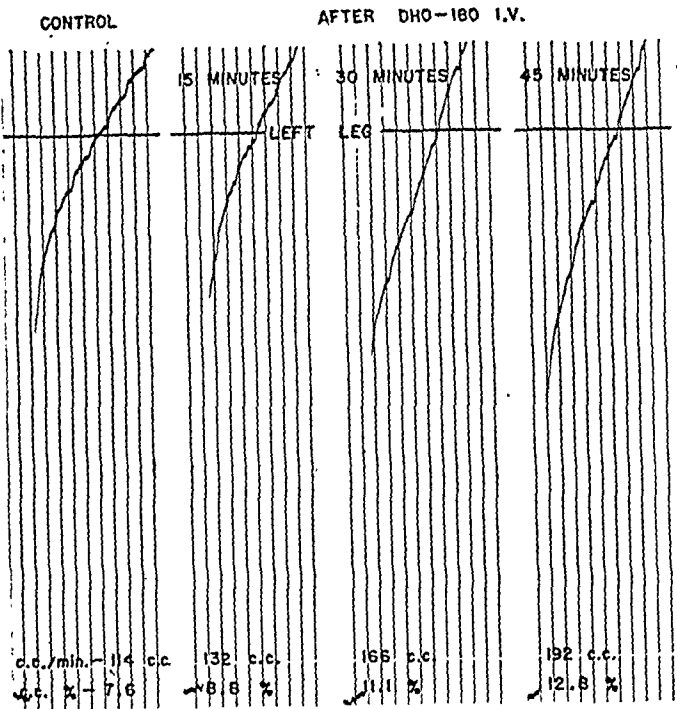


FIG. 2, a

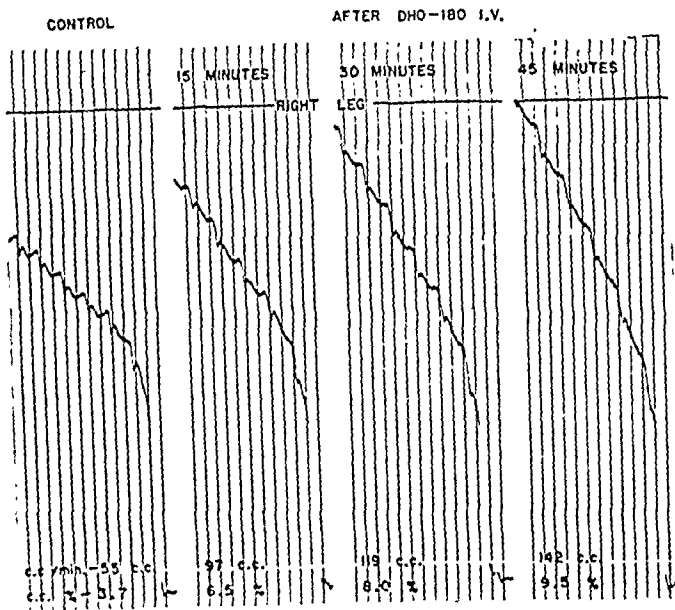


FIG. 2, b

FIG. 2, a and b. REPRESENTATIVE BLOOD FLOW CURVES BEFORE, AND 15, 30 AND 45 MINUTES AFTER THE INTRAVENOUS ADMINISTRATION OF DIHYDROERGOCORNINE

a, left leg; b, right leg.

The curves for the right leg are mirror images of those for the left (see explanation under Figure 1).

of the 20 patients were taken before and at regular intervals after dihydroergocornine had been administered. There was an increase in the amplitude of the pulse in every case—in only one was

the increase considered slight. Figure 4 shows the changes in blood pressure, heart rate and blood flow in a representative case.

COMMENT

The mechanisms by which dihydroergocornine exerts its effects are as yet not entirely clear.

EFFECT OF DIHYDROERGOCORNINE ON DIGITAL PULSE VOLUME

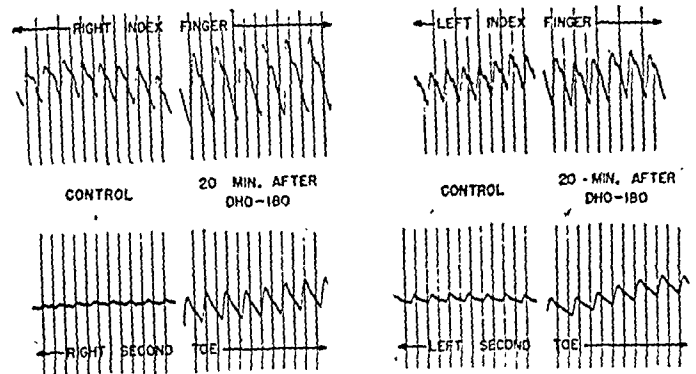


FIG. 3. FINGER-PULSE AND TOE-PULSE VOLUME RECORDINGS BEFORE AND 20 MINUTES AFTER INTRAVENOUS INJECTION OF DIHYDROERGOCORNINE

Note the increase in pulse volume after the drug had been given.

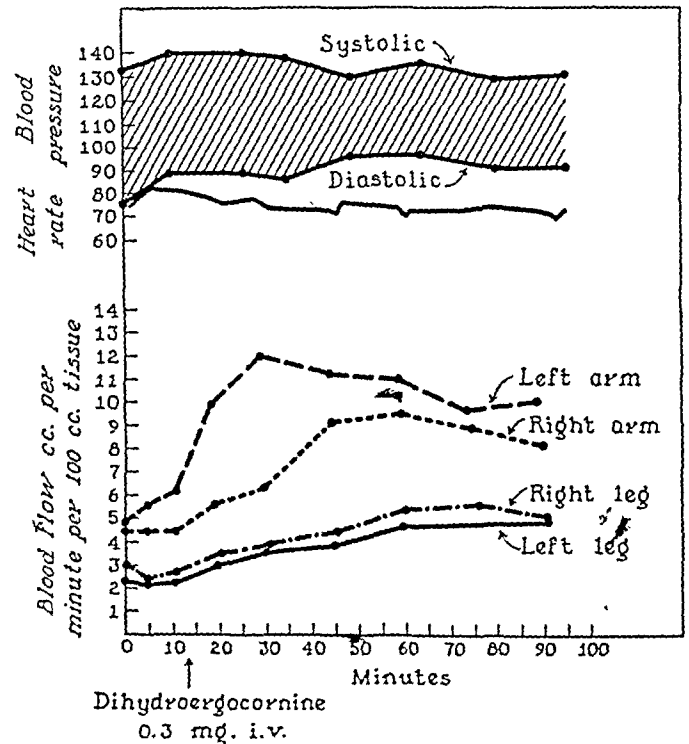


FIG. 4. BLOOD PRESSURE, HEART RATE AND PERIPHERAL BLOOD FLOW BEFORE AND AFTER DIHYDROERGOCORNINE WAS GIVEN INTRAVENOUSLY

Rothlin (11) stated that dihydroergocornine has central and peripheral effects. Its effect on lowering body temperature is of central origin, since it may be blocked by general anesthesia. He further stated that the dihydrogenated alkaloids have a constrictive effect on isolated arteries, blood vessels in the rabbit's ear and in the extremities of the frog. However, in addition to this direct effect on the vessels, there is a latent central sympatholytic action. He did not observe any visible or latent effects on the isolated heart.

In pithed animals the blood pressure is raised by direct vasoconstrictive action on the vessels, but in the intact animal the blood pressure is lowered and there is a reversal of the epinephrine effect after administration of dihydrogenated alkaloids. Thus the action of dihydroergocornine on the blood vessels in animals is bivalent in that central and peripheral mechanisms are present and act in competition with each other. The fall of blood pressure produced by the natural alkaloids is related to the inhibition of the vasomotor center; the fall in blood pressure produced by the dihydrogenated alkaloids is attributed to stimulation of a vasodilator center. Rothlin (11) attributed the decrease in heart rate to central stimulation of the vagal center.

As is seen from the findings in this study, dihydroergocornine produced an increase in the blood flow in the extremities in all but one case. The increase in blood flow in the extremities is indicated by the plethysmographic findings as well as by the increase in the amplitude of the pulse tracings.

From the studies presented here, no accurate statement can be made as to the site of action of dihydroergocornine. Bluntschli and Goetz (5) presumed the site of action to be "in the medulla and/or hypothalamus."

Side effects in our series were more common than previously reported (1-3, 5, 6) and occurred with lower dosages. Because of this fact we decided against the use of doses larger than 0.4 mg.

Conclusions about cumulative and toxic effects of the drug cannot be made from this study since only three patients were given the drug more than once.

SUMMARY AND CONCLUSIONS

The effects of the intravenous administration of dihydroergocornine, an alkaloid of ergot, on the blood pressure, heart rate and peripheral blood flow were studied on 20 human volunteers. The drug was administered to six patients by intravenous infusion of a solution containing 0.5 mg. of dihydroergocornine per 100 cc. of physiologic saline solution, and to 14 patients by a single intravenous injection. The total dose varied from 0.25 mg. to 0.4 mg. The blood flow was determined by means of a venous occlusion plethysmograph with the compensating spirometer recorder.

Dihydroergocornine produced an over-all average increase in peripheral blood flow of 94 per cent in the upper extremities, and 68 per cent in the lower extremities. In one case the blood flow in the upper extremities decreased 3 per cent.

The blood pressure fell in the two hypertensive cases after administration of dihydroergocornine. In normotensives there was no significant change in blood pressure.

The heart rate decreased in every case with an average reduction of 13 beats per minute.

Side reactions were more common than previously reported even with lower dosage. Nasal congestion, nausea, headache, flushing, an urgency for urination and vomiting were the side reactions observed.

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STUDIES OF COPROPORPHYRIN. IV. THE PER DIEM EXCRETION AND ISOMER DISTRIBUTION IN THE URINE IN INFECTIOUS HEPATITIS, INFECTIOUS MONONUCLEOSIS, AND MECHANICAL JAUNDICE¹

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The occurrence of increased amounts of porphyrin in the urine in cases of liver disease was first duly emphasized by Archibald Garrod (1, 2). Garrod believed this to be hematoporphyrin, a confusion with coproporphyrin which was corrected by H. Fischer and Zerweck (3), and which undoubtedly depended upon the very close similarity of the absorption spectra of the two porphyrins. When examined with the relatively crude spectroscopes available at the time of Garrod's study, these porphyrins were indistinguishable. The investigations of H. Fischer, however, made it clear that hematoporphyrin does not occur in the urine, if, indeed, it occurs as a natural product at all. Studies of urinary porphyrin excretion in liver disease, subsequent to that of Garrod, have been for the most part of rather fragmentary nature, consisting of one or not more than a few examples of any one affection. The pertinent literature has been reviewed within recent years (4, 5) and need not be considered here. Attention may be drawn, however, to the papers of Nesbitt and Snell (6, 7) and Localio and co-workers (8), in which considerable data were reported on the feces-urine ratio of coproporphyrin both in obstructive jaundice and liver disease. The papers of Watson (9, 10) and Dobriner (11) present limited data on

the urinary isomer distribution (ratio of types I and III) in these conditions. Dobriner made several important observations in cases of catarrhal jaundice, obstructive jaundice, and cirrhosis of the liver, to which reference will be made again, in this and subsequent papers of this series. Studies of isomer distribution have been generally discouraged, however, by the lack of a method of isomer analysis applicable to small volumes of urine, and as a corollary of this, the lack of exact knowledge of the isomer distribution in normal urine. In previous papers a suitable method has been described (12, 13) and in paper I of this series (14) the results of the application of this method to normal urine have been given. The purpose of the present investigation was to determine the total urinary coproporphyrin (UCP) as well as the isomer distribution in 24-hour samples from cases of infectious hepatitis at various stages of the disease, and also, for purposes of comparison, from cases of infectious mononucleosis and of mechanical jaundice, the latter term being used to designate jaundice due to extrahepatic biliary obstruction, or obstruction of the main hepatic ducts in the liver. It was also desired to assay the value of the UCP determination, relative to other methods of studying liver function, as an indication of residual hepatic functional impairment in cases of hepatitis at various periods after the disappearance of jaundice.

MATERIAL AND METHODS

The method of Schwartz and associates (13) was used to determine the UCP and the isomer distribution. Recent evidence indicates that the true value for the UCP is approximately 20 per cent higher than that obtained with this technique. Nevertheless, all of the data given in the following may be compared directly with the normal data given in paper I (14).

The fractional serum bilirubin was determined according to the modification of the Malloy-Evelyn method

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described by Ducci and Watson (15). The bromsulfalein retention in the blood was determined according to Mateer (16), 45 minutes after injection of 5 mg. of the dye per kilo of body weight. The cephalin cholesterol flocculation test was performed according to Hanger (17). Cholesterol and cholesterol esters were determined by the method of Sperry and Schoenheimer (18). In some instances the urine urobilinogen was determined in 24-hour urine samples (19); in others the quantitative urine Ehrlich reaction (20, 21) was carried out on individual or two-hour samples. The number of coproporphyrin determinations and the cases which were studied are given in Table I.

As noted in Table I, the determinations were often repeated, either with relation to stage of the disease or fluctuation of the jaundice, or in some instances within a brief interval, for purposes of corroboration. The majority of determinations in the infectious hepatitis group were in the period after the disappearance of jaundice.

RESULTS

The data obtained in the five groups of cases (as noted in Table I), are given in Tables II-VI, respectively. The data in Table II permit some comparison of the UCP in cases of hepatitis, with the results of other tests of liver function, as well as

TABLE I

Composition of clinical material and number of determinations

Group	Diagnosis	Number of cases	Number of determinations of UCP	Isomer analyses
1	Infectious hepatitis*	96	172	53
2	Infectious mononucleosis†	25	25	16
3	Cancer of pancreas or bile ducts	41	46	38
4	Common duct stone or stricture	30	49	30
5	Cirrhosis (all types)‡	48	95	53

* This group includes cases of epidemic hepatitis and homologous serum jaundice, some of which were studied in Minneapolis, and some at the Schick and DeWitt General Hospitals of the U. S. Army, at Clinton Ia., and Auburn, Cal., respectively; the urine porphyrin studies were carried out in all instances in Minneapolis. The group also includes cases of epidemic and homologous serum jaundice, and of sporadic hepatitis studied in Minneapolis.

† These cases were studied on the Health Service of the University of Minnesota Hospital, Dr. Ruth Boynton, Director. Liver function studies in this group have been reported elsewhere by Dr. Ralph Peterson (26).

‡ The cirrhosis data will be considered in detail in the ensuing paper of this series, but are included in the present communication for purposes of comparison, with particular respect to the differential diagnosis of jaundice.

UCP in Relation to Duration of Jaundice and Interval after Disappearance of Jaundice

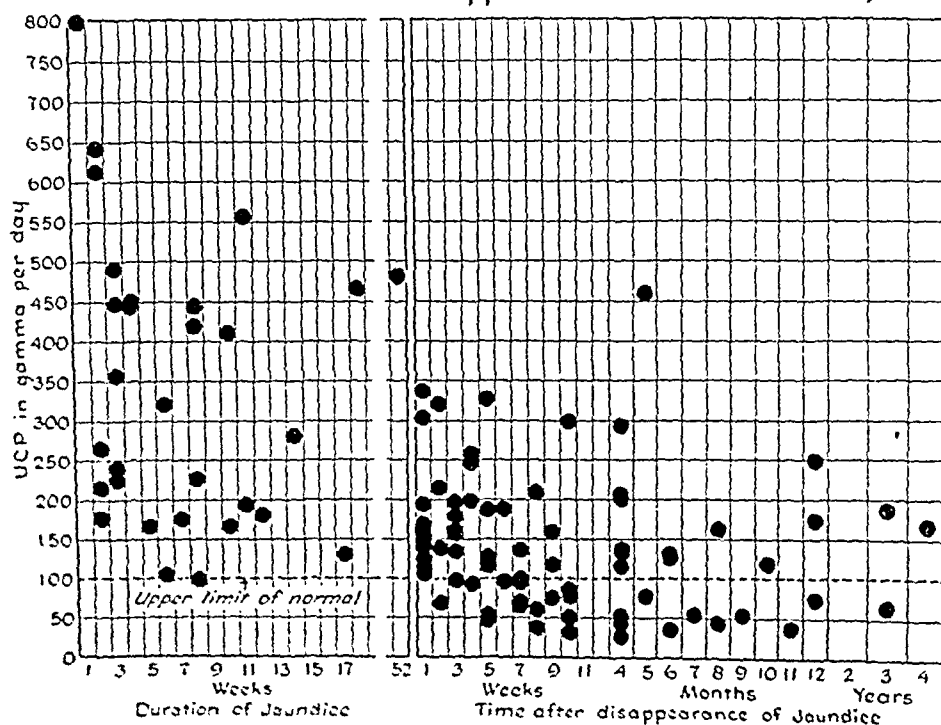


FIG. 1

TABLE II

UCP in cases of infectious hepatitis, compared with stage of disease, symptoms, and status of the liver

Case no.	Initials	Sex	Age	Date	Week of disease	Week after disappearance of jaundice	Symptoms*	Liver enl.†	Tenderness	SB I'	SB T	Brom	CC	CE per cent	UE	UU 24 hr.	UCP $\gamma/24$ hrs.
1	T. M.	♂	30	10-10 11-21	10 16	2 8	0 0	10 3	+	0.7	1.1		3+ 2+	52 66	0.6		320 60
2	J. R.	♂	28	10-20 11-21	4 10	Never j Never j	I 0	0 1	+	0.0	0.03	0 0	1+	40			143 86
3	F. F.	♂	31	10-9 11-15	34 39	5 10	I I	3 3	+	0.0	.44	3 0	0 0	54	.25		47 77
4	R. V.	♂	27	10-10 11-20	11 17	8 14	I I	1 1	0 0	.03	0.1	4		80			209 115
5	H. M.	♂	23	11-16	22	14	0	4	0	.2	.65	24	3+	67	3.8		208
6	F. R.	♂	26	10-9 11-16	6 11	j j	0 0	3 3	+	3.7 5.8	4.1 9.2		1+		0.7- 3.0		319 193
7	M. D.	♂	23	10-1 11-15	13 19	7 13	I I	4 5	+	0.0	0.17	1	0	57	1.8		65 292
8	J. T.	♂	35	10-7 11-16	5 11	1 7	++ ++	4 6	0 ++	0.3	0.7	4	0	63			193 99
9	H. C.	♂	37	9-20 10-27	25 30	9 14	++ 0	2 1	+	0.1	0.2	0 0	0 0	45 56	2.5		159 133
10	R. C.	♂	28	9-27 11-21	11 18	7 14	I +	3 2	0 0	0.5	1.1	9 5	0		1.5		138 28
11	P. U.	♂	21	10-2 11-16	4 10	j 5	+	2 0	0 0	2.7	4.3		4+		2.3 0.5		444 115
12	J. R.	♂	21	10-8 11-15	3 8	j sl j	++ I	4 1	+	3.1	5.5	0	1+	36	3.8		448 99
13	A. M.	♂	21	10-1	24	4	0	0	0	0.1	0.2	8	0	47	1.8- 4.6		92
14	C. M.	♂	37	9-20 11-15	36 39	30 33	++ I	5 0	++ 0	0.3	0.6		0	69	2.0- 2.7		161 52
15	W. W.	♂	40	9-24	56	46	I	1	0	0.0	0.3	0	0	61	0.7		172
16	F. H.	♂	40	9-20 11-15	3 yr. 10 wk. 3 yr. 14 wk.	Approx. 3 yr. Approx. 3 yr.	+	0	0	0.2	0.44	0	0	49	1.1- 3.3		187 63
17	M. H.	♂	21	9-28	4	2	0	4	0	0.5	0.65	15	0		0.5		213
18	J. H.	♂	23	9-20	1 yr. 3 wks.	6	+	3	+	0.0	0.2	0		60	2.0		189
19	W. M.	♂	22	9-28	10	3	0	2	0	0.2	0.58	5	0		1.0		96
20	J. D.	♂	32	10-2	19	17	0	0	0	.23	.44		0		2.3		76

* Symptoms: I = Indefinite or questionable; +, ++ mild or moderate weakness, fatigue, anorexia; +++ severe anorexia and weakness, patient very sick but not comatose.

† Enl.: Cm. below costal margin in midclavicular line. Tenderness: + = slight, ++ = moderate, +++ = marked.

Key: SB = Serum bilirubin

I' = prompt direct

T = total

CE = Cholesterol ester %

Brom = % retention of Bromsulfalein in blood 45' after 5 mg. per kilo body weight

UE = Urine Ehrlich units per 2-4 P.M. urine sample

CC = Cephalin cholesterol flocculation at 24 hours

UU = mg. urobilinogen in 24 hr. urine

TABLE II—Continued

Case no.	Initials	Sex	Age	Date	Week of disease	Week after disappearance of jaundice	Symp-toms*	Liver enl.†	Tender-ness	SB 1'	SB T	Brom	CC	CE per cent	UE	UU 24 hr.	UCP [‡] 7/24 hrs.
21	P. B.	♂	30	10-1	8	1	I	6	+	0.1	.52				1-2.2		159
				11-15	14	7	I	5	+	0.1	.38	0	0	56	0.5		94
22	R. G.	♂	28	9-28	10	sl j	0	10	0	1.7	3.3	9	3+	22	1.8		167
				11-16	16	5	0	0	0	0.2	0.7	5	0	74	0.6		52
23	D. H.	♂	26	9-26	18	10	0	0	0	.03	.4	1	2+		4.6-1.2		150
24	J. M.	♂	23	10-2	13	2	+++	4	+++	0.3	.8	5	0	52	1.9		67
				11-15	19	8	0	0	0	.17	.52	2.5	0	58	1.2		37
25	V. N.	♂	24	10-1	9	7	0	0	0	.03	.23						69
				11-21	15	13	0	0	0								51
26	W. R.	♂	24	10-1	28	24	+	0	+	.03	.44		0		.8		127
					34	30	0	0	0								43
27	J. S.	♂	33	9-28	12	4	0	0	0	0.1	.23	12	0	49	1.4		256
				11-15	18	10	0	0	0			0					32
28	J. W.	♂	24	9-28	28	2-4	+	3	0	0.5	1.5	10	0	51	5.0		197
				11-16	34	8-10	I	0	0						.3-1.6		116
29	R. F.	♂	25	11-15	52	28	0	2	0	0.01	0.41				0.97		53
30	E. P.	♂	28	2-16	4	j	+++	6	+++	17.0	30.7		3+	15	1.8-5.0	15	450
				3-26	10	j	I	5	0	2.13	5.13		1+	62	2.8		410
				4-14	12	1	0	5	0	0.5	1.75				4.95		336
31	E. C.	♂	22	2-16	13	3	+	0	0	0.5	1.6	1.5	0		0.5		132
32	R. B.	♂	24	2-17	3	j	I	0	0	1.0	3.2	7	1+	79	1.2		240
				3-20	7	3	0	0	0	0.4	1.2	0.5	2+		1.7		160
33	W. M.	♂	23	4-4	5	2	0	2.5	0	0.5	1.6	4	0	74	2.7		139
34	R. B.	♂	30	2-16	3	j	+++	5	0	10.9	19.5		3+		4.5		490
35	R. R.	♂	35	4-4	28	16	+	3	++	0.1	1.1	0	0	76	1.8		47
36	C. M.	♂	37	2-16	4	1	0	0	0	0.6	1.3	0	0	71	2.2		150
37	L. H.	♂	25	3-4	23	10	++	8	++	0.1	0.99	0.5	0	63	0.73		86
38	H. J.	♂	25	4-4	26	15	0	0	+	0.25	1.1	3.5	0	76	.96	1.5	136
39	W. C.	♂	25	4-4	10	1	I	1	0	0.65	1.65		1+		2.1		152
				4-14	12	3	0	0	0	0.4	0.95	1	0		1.0		180
40	C. R.	♂	27	3-26	1 yr.	j	+++	5	0	8.0	16.0		3+	39	8.0		480
41	J. B.	♂	45	2-5	6	4	I	0	0	0.5	1.0	12	1+	53	12.0		198
				2-16	7	5	I	0	0	0.5	1.1	15	2+		2.2		328
				3-13	11	9	0	0	0	0.2	0.8		1+	70	0.9		75
42	F. D.	♀	44	11-9-16	5	j	++	2	0	12.8	22.2		4+	25	0.1		164
				11-14	6	j	++	2	0	12.8	24.3		4+	31			104
				11-24	7	j	++	2	0	13.5	25.6		4+	25	0.4		156
				11-27	8	j	++	2	0	12.7	25.1		4+	20	1.2		225
43	E. G.	♂	48	8-9-16	12	j	+	0	+	2.5	4.8		0	68	1.6		180
				8-22	14	j	+	0	+	1.4	2.8	1.4	1+	69	0.5		280

TABLE II—Continued

Case no.	Initials	Sex	Age	Date	Week of disease	Week after disappearance of jaundice	Symptoms*	Liver enl.†	Tenderness	SB 1'	SB T	Brom	CC	CE per cent	UE	UU 24 hr.	UCP 7/24 hrs.
44	B. K.	♂	32	5-15-46 6-1 6-2 7-10	18 20 20 25	j 1 1 6	+ + + +	5 3 3 3	+ + + 0	1.9 0.9 0.2 0.5	4.5 1.8 0.5		3+ 1+	76 67	0.9 0.6		465 134 164 94
45	A. G.	♂	21	3-12-46 3-17 5-2 10-16	19 20 26 48	16 17 23 45	+ 	2 2 0 0	+ + + 0	0.2 .06 0.1	1.0 0.8 0.7	3 0 0	0 74 0	73 74	0.7 0.5	10.4 1.0	203 460 129 249
46	A. G.	♂	40	4-16 4-24	1 3	j sl j	+ 	2 0	0 0	4.1 0.9	7.1 1.7		4+ 4+	75		22.3	779 356
47	A. C.	♀	28	4-13	3 yr.	1 yr. 2 mo.	+	1	++	0.1	0.7	3%	0	75	1.7		72
48	C. M.	♂	30	5-26	8	j	I	1	+	26	37.9		0		4.6		420
49	V. A.	♂	41	2-24 3-15 4-8 5-6 6-17 8-8	8 11 14 18 23 30	j j 1 5 10 17	+ + 0 0 0 0	2 2 0 0 0 0	+ 0 0 0 0 0	5.9 2.6 0.6 0.3 0.2 0.2	10.1 4.6 1.5 0.8 0.6 0.6		0 2+ 0 0 0	55 69		11.1 26 2.7 2.9 0.7 1.2	445 554 302 188 259 253
50	M. B.	♀	30	12-1	1 yr.	About 11 mo.	I	0	0	0.2	2.1	2%	1+	74	0.6	1.0	36
51	L. H.	♀	32	9-4 9-7 9-24	2 2 3	j j 1	+ + 0	1 1 0	+ + 0	1.8 .16	3.0 0.7	30.5 3	4+ 4+	35		20.0	265 642 148
52	M. H.	♀	27	2-28	16	1	+	1	+	0.8	1.7	12.5	3+	60		2.6	116
53	I. S.	♀	52	12-4	17	j	+	0	0	1.2	2.2		2+	67		0.7	130
54	G. B.	♂	30	5-20 7-8	31 37	13 19	+ 0	+ +	0 0	0.2 0.2	2.0 1.8	2.4 2.0	0 0			4.2 0.3	143 135
55	A. B.	♂	40	4-30-46	28	22	+		0	0.2	2.8	5.0	1+		1.9		35
56	P. T.	♂	54	4-28-46 6-7-46	2 7	j 1	+ +	+ 0	+ 0	15.4 0.6	26.7 1.2		3+ 0	26 67	10.0 3.2		611 105
57	G. T.	♂	50	4-19-48 5-8-48	2 4	j 4	+ +	2 2	+ +	4.6 14.7	7.7 31.7		4+ 4+	50.5 21	11.2	1.0	224 245
58	M. S.	♀	19	7-17-47	2	Never j	+	+	+	0.2	0.5	2	1+			1.5	45
59	H. F.	♀	37	7-2-47	4 yr.	4 yr.	+	0	0	0.2	1.1	3	0		4.5		153
60	L. S.	♀	47	5-2-45	2	j	+	1	+	9.0	13.0		3+	17			178
61	R. G.	♂	21	7-1-45	12	10	+	1	+	0.1	0.5		0			0.3	119
62	H. H.	♂	50	3-18-48 3-29-48 4-11-48 4-25-48	3 5 7 9	j 1 3 5	+ + + 0	2 1 1 0	+ + + 0	1.3 0.4	2.5 1.1		3+ 0 0 0	67		1.3 1.0 1.0	222 141 158 125

with the patients' symptoms, approximate liver size, and degree of tenderness, at various stages of the disease. Table IIA includes supplementary data on the UCP; in these instances additional information as to hepatic function was lacking or fragmentary.

In Figure 1, the values for UCP in the cases of hepatitis are plotted with relation to the duration of jaundice or the interval from the disappearance of jaundice. This figure includes all of the determinations in all cases in which there had been jaundice. As a matter of fact, the present mate-

TABLE IIA

Additional UCP determinations in cases of infectious hepatitis

Case no.	Initials	Age	Sex	Presence or absence of jaundice	UCP in $\gamma/24$ hrs.
63	J. B.	67	♀	+	257
64	A. P.	29	♀	-	206
65	E. S.	30	♂	-	739
					448
				+	425
					472
					220
					155
				-	134
					91
					158
66	C. R.	31	♂	-	197
67	E. R.	24	♂	-	39
					42
				-	77
					120
					37
68	J. M.	59	♂	+	432
69	L. S.	42	♂	+	451
70	I. N.	24	♂	+	252
71	C. L. F.	23	♂	+	346
72	O. D.	54	♂	+	205
73	L. F.	45	♂	+	242
74	S. S.	55	♂	+	265
				-	235
75	M. A.	25	♂	-	151
76	J. M.	21	♂	+	170
77	W. S.	23	♂	+	312
78	N. T.	21	♂	+	197
79	J. O.	34	♂	+	222
80	H. W.	42	♂	+	505
81	D. W.	27	♂	-	150
82	R. S.	23	♂	+	241
83	R. K.	24	♂	+	314
84	B. R.	30	♂	+	296
85	P. T.	21	♂	+	210
86	C. S.	26	♂	-	160
87	J. G.	27	♂	-	208
88	F. W.	4	♂	+	179
89	M. G.	25	♂	-	141
90	R. F.	25	♂	-	540
91	B. R. K.	22	♂	+	177
92	H. F.	53	♂	-	145
93	G. G.	23	♂	-	210
94	T. C.	40	♂	+	304
95	C. B.	74	♂	+	279
96	E. E.	20	♀	+	182

rial includes but one instance of what was believed to represent hepatitis without jaundice (Case 2 in Table II). The upper limit of normal of 100 γ , as indicated in Figure 1, was established with relation to the value of the mean plus two standard deviations, or 97 γ . One instance of the 53 in the normal group was above this value, with 99 γ (see paper I of this series [14]).

As shown in Figure 1 and Table II, a number of the cases of hepatitis were first seen in a late stage of the disease, weeks or months after the

TABLE III

Coproporphyrin isomer distribution in 24-hour urine samples from cases of hepatitis

Case no.	UCP in $\gamma/24$ hrs.	Isomer distribution in per cent		Type III $\gamma/24$ hours
		I	III	
4	209	88	12	25.1
6	319	92	8	25.5
8	193	92	8	15.4
10	138	84	16	22.1
12	488	96	4	19.5
14	161	81	19	30.6
15	172	80	20	34.4
19	96	90	10	9.6
23	150	90	10	15.0
26	127	87	13	16.5
27	256	93	7	17.9
30	450	98	2	9.0
32	160	93	7	11.2
34	490	99	1	4.9
36	150	93	7	10.5
38	136	92	8	10.9
39	152	91	9	13.7
40	480	96	4	19.2
41	198 328 75	88	12	23.8
		90	10	32.8
		87	13	9.8
42	164 106 156	92	8	13.1
43	180 280 465	89	11	24.8
		90	10	18.0
		86	14	39.2
44	134 164 94	95	5	23.3
45	203 460 129	89	11	18.0
46	249 779 356	93	7	32.2
		90	10	12.9
47	72	96	4	31.2
48	420	86	14	10.1
49	445	91	9	37.8
53	130	90	10	44.5
54	143	91	9	11.7
54	120	91	9	12.9
67	120	85	15	18.0
69	415	89	11	9.8
72	205	80	20	41.0
75	151	79	21	31.7
79	222	90	10	22.2
84	296	90	10	29.6
86	160	91	9	14.4
87	208	94	6	16.5
88	179	83	17	30.4
89	141	86	14	19.7
90	540	96	4	21.6
91	177	92	8	14.2
92	145	85	15	21.8
93	210	95	5	10.5
94	304	89	11	33.4
95	279	98	2	5.6
96	182	86	14	25.5

disappearance of jaundice. At least two of these are sufficiently instructive to be given in more detail in the following:

TABLE IV
UCP and isomer distribution in cases
of infectious mononucleosis

Case	UCP in $\gamma/24$ hrs.	Isomer distribution in per cent		Type III in $\gamma/24$ hrs.
		I	III	
1. D. J.	189	88	12	23
2. B. R.	163	81	19	31
3. M. G.	410	96	4	16
4. L. V. B.	147	80	20	29
5. G. B.	364	89	11	40
6. H. S.	111	82	18	20
7. E. S.	425	92	8	34
8. P. C.	123	82	18	22
9. C. M.	282	93	7	20
10. E. B.	133	80	20	27
11. E. F.	249	95	5	12
12. E. W.	113	90	10	11
13. D. S.	219	85	15	33
14. F. K.	113	90	10	11
15. T. G.	143	88	12	17
16. D. E.	152	92	8	12
17. F. S.	46			
18. S. A.	133			
19. R. G.	71			
20. D. D.	45			
21. C. A.	177			
22. A. H.	42			
23. K. M.	73			
24. C. D.	77			
25. G. L.	122			

1. Case 45, A. G., male 21, farmer. The patient was first seen at the University Hospital on March 11, 1946. He stated that about six months earlier he developed nausea and anorexia, shortly followed by jaundice which persisted for two months. During the last four months he felt fairly well and had resumed his work, but was bothered by persistent discomfort and tenderness in the right upper abdomen. Examination revealed an enlarged tender liver and a palpable spleen. Routine laboratory studies were normal. As seen in Table II, the fractional serum bilirubin, thymol turbidity, cephalin cholesterol flocculation, bromsulfalein, cholesterol and ester percentage, were all within normal limits. The serum proteins, hippuric acid synthesis, and prothrombin time were also normal. The urine Ehrlich test on individual samples was normal, but the 24-hour urine urobilinogen was 10.4 mg., in other words, considerably elevated. The UCP was markedly increased, usually above 200 $\gamma/24$ hours (see Table II). The liver biopsy revealed evidence of an active hepatitis, *i.e.*, small foci of cellular exudate with occasional necrosis, but no fibrosis.

2. Case 49, V. A., male, 41, illustrator. This

case is presented in more detail because of the unusually favorable opportunity for study that was presented during the latter weeks of a prolonged "catarrhal" jaundice or sporadic infectious hepatitis. Following is a brief summary of the salient clinical features:

The first day of observation, Figure 2, was February 24, 1947. At this time the patient had been jaundiced for two months. On December 7, 1946, he experienced an acute attack of diarrhea, nausea and vomiting, of 12 hours' duration. He recovered and felt quite well, but on December 26, he noted fever, malaise, and pain in the muscles. His urine became dark, and he consulted a physician who made a diagnosis of infectious jaundice. When first seen at the University Hospital the liver was moderately enlarged and tender. Within three weeks the tenderness had disappeared and the jaundice had markedly diminished. The liver was still palpable. By April 8, the jaundice had disappeared though the serum bilirubin was still distinctly elevated. Although the patient's appetite had returned, a moderate lassitude persisted throughout the summer. He did not have energy enough to play golf which he ordinarily enjoyed. By November of 1947, however, he believed that he had regained an entirely normal status. As noted in Figure 2, the results of the liver function studies, except the UCP, were normal by the 65th day of observation (corresponding to April 30, 1947). The UCP, however, remained considerably elevated, the value on the 171st day (August 14, 1947), being 253. Later determinations have continued to show abnormally high values, as seen in Table VII. The patient feels quite well (December 15, 1948) but has now developed a number of small spider nevi over the neck, upper chest, and hands. The liver is palpable at the costal margin, not tender.

The total coproporphyrin values together with the percentage and actual amount of each isomer as determined in 46 cases of infectious hepatitis, are given in Table III. Similar data for the infectious mononucleosis group are shown in Table IV. In Figure 3, the initial values obtained for the total UCP and per cent of type III isomer, in the first 40 cases of hepatitis to be studied in this way, are plotted in comparison with the values for the normal (Minneapolis) series previously reported (14), and for 38 cases of acute poliomyelitis.

UCP and Other Liver Function Studies
in Subsiding Infectious Hepatitis

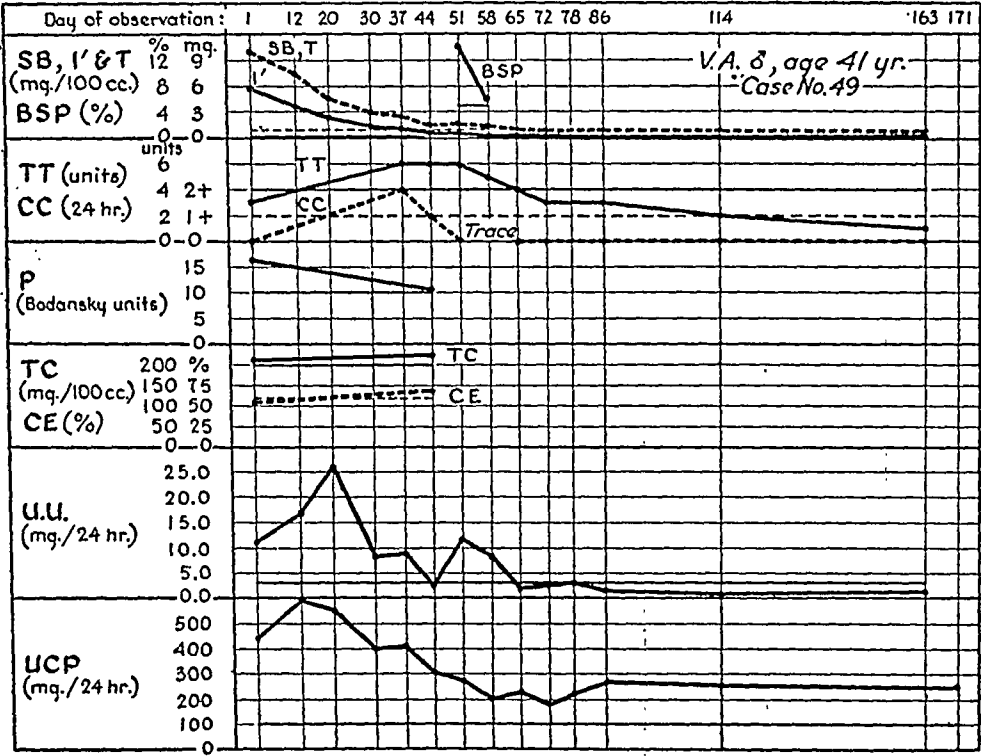


FIG. 2

UCP and Percentage of Type III Isomer
in Poliomyelitis and Infectious Hepatitis

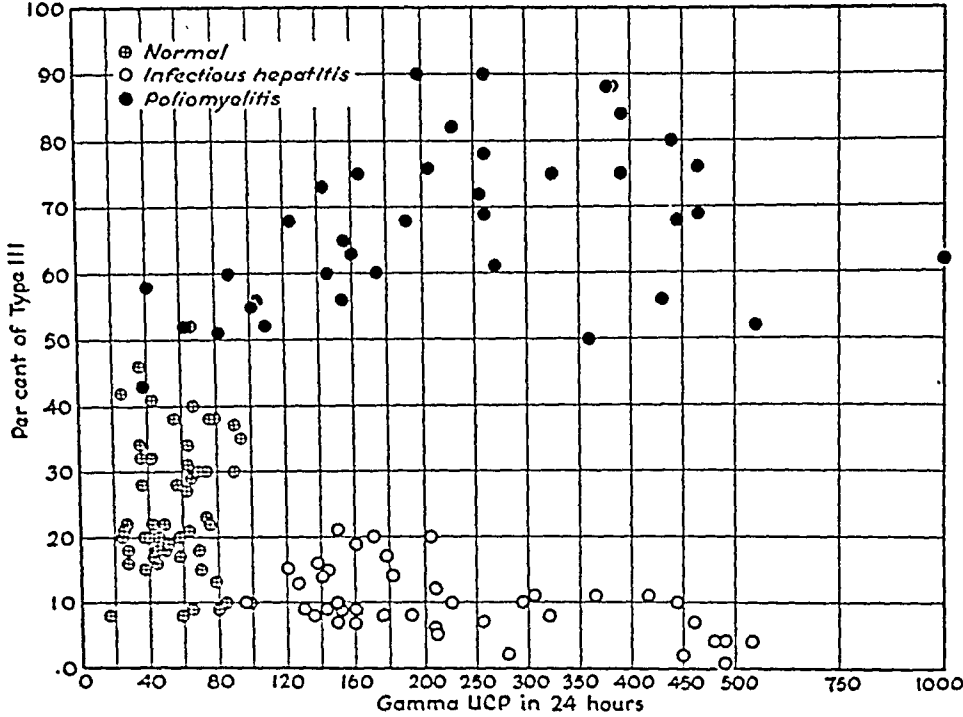


FIG. 3

The latter data have also been reported previously (22) and are included here only for purposes of comparison with the results in the hepatitis cases. One instance was of particular interest because of the likelihood of coexistence of infectious hepatitis and poliomyelitis.

Case 51, L. H. female, 32, housewife. This patient was seen during the epidemic of poliomyelitis in 1946. She complained of headache and stiff neck, and there was fever. The spinal fluid was found to contain 17 mononuclear cells per cu. mm. When first seen, there was distinct jaundice, in ad-

dition to the findings indicative of poliomyelitis. A history was obtained of painless jaundice suffered by her son and several individuals in the neighborhood about six weeks earlier. The patient had observed dark urine at the outset of her own illness. The liver was not palpable or tender at any time. The laboratory data as seen in Table II revealed marked liver functional impairment. The first UCP on September 4, was 265 γ /24 hours; three days later it was 642 γ , and at this time 82 per cent was type I, the remainder type III. This represents 116 γ of type III. In paper I of this

TABLE V
UCP in cases of cancer of the pancreas, bile ducts, or liver

Case no.	Initials	Sex	Age	Diagnosis	Diagnosis by*	Date of determination	SB†		UCP γ /24 hrs.	Per cent I	Per cent III	γ III/24 hrs.
							I'	T				
1	C. K.	♀	56	Ca. bile ducts	A	8/27/44 8/29/44 8/30/44	22.0	25.5	330 269 380	95	5	17
2	J. W.	♂	51	Adeno ca. of bile ducts	O	3/2/46	12.8	23.0	314	89	11	35
3	H. A.	♂	74	Ca. C.B.D.	O	12/6/46	16.5	23.2	257			
4	H. A.	♂	59	Ca. C.B.D.	O	7/18/46	24.0	41.6	226	81	19	43
5	G. H.	♀	50	Ca. C.B.D.	O	5/27/46 5/29/46	15.1	31.2	381 172	94	6	23
6	O. O.	♂	53	Ca. head of pancreas	O	4/26/46	3.9	6.5	365	86	14	51
7	E. P.	♂	77	Ca. body of pancreas	O	12/10/45 12/11/45 12/22/45	6.6	9.6	201 180 144	78	22	51
8	G. S.	♂	76	Adeno ca. of bile ducts	O	12/25/46	13.8	24.0	174			
9	W. G.	♂	70	Ca. pancreas	A	4/17/47			196	82	18	36
10	J. D.			Ca. pancreas	A	5/6/47			206	80	20	
11	J. W.	♂	67	Ca. pancreas	O	11/18/44	9.1	15.8	281	91	9	25
12	C. A.	♀	71	Ca. pancreas	C	1/18/48	15.6	27.9	188	82	18	34
13	E. B.	♂	65	Ca. pancreas	A	5/17/47	16.8	31.0	167	85	15	25
14	S. B.	♂	55	Ca. C.B.D.	O	10/9/44	8.0	15.1	282	80	20	56
15	E. C.	♀	62	Ca. G.B.	O	6/14/47	15.3	26.0	364	75	25	91
16	C. C.	♂	75	Ca. liver	O	4/6/47	0.1	0.3	331	74	26	86
17	C. C.	♂	56	Ca. pancreas	O	9/24/47	14.8	28.0	282	62	38	107
18	A. D.	♂	54	Ca. body pancreas	O	2/7/47	0.1	0.4	66	81	19	12.5
19	H. E.	♂	67	Ca. pancreas	A	12/20/44	28.5	43.0	217	74	26	56.5

* A = autopsy
O = operation
C = clinical study only

† SB = serum bilirubin
in mg. per 100 cc.
I' = prompt direct; T = total

TABLE V—*Continued*

Case no.	Initials	Sex	Age	Diagnosis	Diagnosis by*	Date of determination	SB†		UCP γ/24 hrs.	Per cent I	Per cent III	γ III/ 24 hrs.
							I'	T				
20	M. E.	♂		Ca. pancreas	O	6/18/47	9.0	15.8	443	71	29	128
21	A. F.	♂		Ca. C.B.D.	O	10/17/47			294	70	30	88
22	P. G.	♂	74	Ca. pancreas	C	8/23/47	24.2	42.1	198	80	20	39
23	H. G.	♂	65	Ca. liver	O	2/27/47			127	69	31	39
24	N. H.	♂	21	Ca. liver	O	6/30/47	0.1	0.5	79	68	32	25
25	L. H.	♂	69	Ca. C.B.D.	A	10/21/47	7.8	14.5	177	77	23	41
26	R. J.	♀	25	Ca. pancreas	O	10/20/47	6.3	11.1	242	72	28	68
27	A. J.	♂	45	Ca. liver	O	4/24/46	0.1	0.9	61	71	29	18
28	H. J.	♀	66	Ca. C.B.D.	A	8/13/46	0.2	0.4	280	74	26	73
29	M. J.	♀	56	Ca. C.B.D.	C	3/3/48	2.7	3.8	149	72	28	42
30	S. K.	♂	73	Ca. liver	C	9/30/46	16.0	30.0	297	81	19	56.5
31	W. R.	♂	70	Ca. C.B.D.	O	2/1/46	6.6	9.8	272	77	23	62.5
32	F. U.	♂	67	Ca. pancreas	C	2/18/48	10.0	16.5	190	78	22	42
33	C. W.	♀	58	Ca. liver	O	7/13/46	0.1	0.8	137	84	16	22
34	L. W.	♀	61	Ca. C.B.D.	O	3/26/47	8.7	14.5	214	70	30	64
35	J. W.	♂	70	Ca. head pancreas	O	3/2/46	4.6	8.7	314	80	20	63
36	H. K.	♂	65	Ca. bile ducts	O	2/4/47	13.7	24.0	204	75	25	82
37	E. J.	♂	60	Adenoca of ampulla	O	8/14/47	5.6	12.2	208	71	29	71
38	N. P.	♂	60	Ca. pancreas	O	5/10/47	0.4	0.9	113	79	21	24
39	C. A.	♀	68	Ca. bile ducts	O	4/9/48	3.6	6.4	175	70	30	53
40	R. S.	♀	53	Ca. bile ducts	C	7/10/48	0.2	1.1	249	81	19	47
41	B. W.	♀	21	Ca. bile ducts	A	1/30/47	5.6	10.2	300	74	26	79

series, it was noted that 31 γ was two standard deviations above the mean for the normal group. Thus it is evident that there may be a marked increase of one isomer, even though, percentagewise, the other one is preponderant.

Tables V and VI include the data for the biliary tract cancer and the calculous groups respectively. Two exceptional cases included in Table VI deserve individual consideration. Both of these exhibited considerable increases of type III isomer in contradistinction to the other members of the group, in which the increase was mainly of type I. In both, however, there were prominent features serving to distinguish them in other respects as well. In Case 2, gallstones were found in a large

sub-hepatic pocket resembling an abscess; the gall bladder and common duct were not identified and the surgeon believed that there had been a severe inflammation with considerable "breaking down of tissue." The spleen, which was removed, weighed 1270 gm., and was diagnosed as splenic hyperplasia or Banti's disease; liver biopsy revealed a definite early cirrhosis. It was impossible to obtain a history of alcoholism or other chemical exposure which might have explained the excessive type III coproporphyrin excretion in this case. The possibility of endogenous chemical intoxication is considered because of the above operative findings.

In Case 12, Table VI, necropsy revealed miliary

tuberculosis. The possible relationship of this condition to an excessive type III coproporphyrin excretion is not clear and is receiving further study. Again one must consider an endogenous chemical intoxication, due to extensive necrosis, as the important factor.

A third case, not included in the tables, and which we have not been able to classify, was that of an aeronautical engineer, a male, 47 years of age, who was conducting research at very low temperatures. In fact, 24 hours before the onset of his illness, he had spent an hour and a quarter in a cold chamber at -60°F , wearing a suit specially designed for high-altitude flying. The temperature of

his skin, measured by means of a thermocouple, fell to as low as 84°F during the latter part of the experiment. He felt fairly well until the next day when he noticed marked aching in the muscles. This persisted and the next day his temperature rose to 103°C . Two days later he became jaundiced; the urine was dark and the stools light. The jaundice became more marked and persisted about two weeks, the maximum serum bilirubin being 6.8 mg. per 100 cc., of which 3.7 mg. was the prompt direct (one minute) type. The cephalin cholesterol flocculation test was 3 plus. The 24-hour urine urobilinogen was 113 mg. The UCP was 270 $\gamma/24$ hours of which 90 per cent was the

TABLE VI
UCP in cases of common duct stone

Case no.	Initials	Sex	Age	Date	SB*		UCP $\gamma/24$ hrs.	Per cent I	Per cent III	γ III/ 24 hrs.	Diagnosis by†
					I'	T					
1	M. S.	♀	70	4/5/43	5.8	8.1	230	88	12	28	O
2	C. M.	♀	46	8/18/44	4.4	7.4	260	35	65	169	O
3	M. R.	♀	32	8/28/44	2.7	4.5	120				O
4	H. P.	♀	50	8/7/46 5/5/44	0.9 13.0	1.7 16.3	183 330	87	13	24	O
5	L. C.	♀	78	1/13/47	0.3	1.5	139				C
6	H. B.	♂	46	11/7/44	0.4	1.2	230	93	7	16	C
7	J. R.	♂	58	11/7/44			202	87	13	26	C
8	E. S.	♀	37	12/15/45	2.3	4.8	175				O
9	A. M.	♂	51	9/16/46 9/17/46 9/18/46 9/19/46 9/20/46 9/21/46 9/22/46 9/23/46	2.3 1.0	3.9 2.0	325 302 330 344 264 199 152 109	92	8	26	O
10	M. R.	♀	62	6/25/46 6/26/46 6/27/46 6/28/46 6/29/46 7/1/46 7/3/46 7/7/46	8.9 3.5	14.2 7.2	509 223 216 206 180 419 218 238	95 92	5 8	25 17	O
11	B. S.	♂	54	4/2/46	0.3	1.0	248	84	16	40	C
12	F. S.	♂	71	5/23/44 5/30/44	4.3	4.9	180 184	17	83	149	A

* SB = serum bilirubin in
mg. per 100 cc.
I' = prompt direct
T = Total

† A = autopsy
O = operation
C = clinical study only

TABLE VI—Continued

Case no.	Initials	Sex	Age	Date	SB*		UCP $\gamma/24$ hrs.	Per cent I	Per cent III	γ III/ 24 hrs.	Diagnosis by†
					I'	T					
13	J. M.	♂	72	9/17/47 9/18/47 9/20/47 9/21/47	4.5	10.0	575 512 263 262	83 82 85	17 18 15	97 92 39	O
14	M. S.	♀	59	1/25/48	1.9	3.8	117	90	10	12	O
15	R. B.	♀	61	1/25/48	0.9	1.6	115	88	12	14	O
16	F. G.	♂	81	4/29/47	2.2	3.7	238	93	7	17	A
17	J. W.	♂	72	7/9/46	0.4	1.4	89	91	9	8	O
18	J. J.	♂	58	3/3/46	3.1	6.5	159	81	19	30	O
19	A. H.	♀	74	9/3/46	8.4	12.9	214	77	23	49	O
20	M. H.	♂	46	12/9/45	0.2	2.1	172	85	15	26	C
21	M. C.	♀	30	5/5/46	0.2	1.7	67	82	18	12	C
22	H. H.	♀	37	7/8/46	0.9	2.6	116	86	14	16	O
23	C. F.	♀	68	1/8/47	1.2	3.0	210	91	9	19	O
24	I. R.	♀	40	5/12/47	0.1	0.4	64	79	21	13	O
25	A. F.	♀	58	9/18/47	0.2	1.1	116	83	17	20	C
26	I. M.	♀	66	4/4/48	0.3	1.3	67	79	21	14	O
27	E. M.	♀	38	1/7/48	0.1	0.9	108	80	20	22	C
28	K. H.	♂	71	4/12/48	0.2	1.1	105	90	10	11	C
29	C. H.	♀	73	2/13/48	2.5	4.9	186	94	6	11	O
30	L. C.	♂	41	11/15/48	0.4	1.4	138	88	12	17	O

TABLE VII

UCP and isomer distribution in Case 49 (Figure 2)

Date	UCP in $\gamma/24$ hrs.	Per cent Type I
2-25-47	445	90
3-7	593	
3-15	554	
3-25	400	
4-2	413	
4-8	302	
4-15	275	93
4-22	201	
4-29	234	
5-6	188	
5-13	225	
5-20	270	
6-17	259	
8-5	122	
8-14	253	
1-29-48	200	
2-8	232	94
2-8	290*	95
7-16	130	
10-29	266	

* Feces—amount for one day.

type III isomer. The attack was relatively mild and the patient felt quite well six weeks later. This would in all likelihood have been accepted as an episode of sporadic infectious hepatitis if it were not for the curious circumstances at the outset, coupled with the result of the porphyrin determination. The former might be regarded simply as a precipitating factor, but it was then found that there had been considerable alcoholism in the weeks or months preceding the onset of the jaundice, also that there had been moderate exposure to a number of chemicals and heavy metals in connection with the patient's research. Furthermore, the excessive type III coproporphyrin excretion persisted long after the attack of jaundice which has just been described. On March 8, 1946, nearly two years later, the UCP was 208 $\gamma/24$ hours with 65 per cent type III; on August 14, 1946, it was 187 γ ,

and on November 23, 1946, 175 γ . Thus, while it was impossible to classify this case, it appeared reasonable to believe that it was more likely due to extrinsic chemical and possibly physical factors, and less likely to an infectious hepatitis, although a combination of chemical and viral factors is a distinct possibility.

DISCUSSION

The present results reveal that the urinary coproporphyrin is quite uniformly increased in regurgitation jaundice, whether due to biliary obstruction or to parenchymal hepatic disease. Further study is necessary to determine if this is due only to regurgitation of bile into the blood stream, or to hepatocellular functional impairment, or to a combination. The persistent and often marked elevation of the urinary coproporphyrin long after the disappearance of jaundice points toward an hepatocellular or retention, rather than a cholangiolar or regurgitation factor. The elevated values in obstructive jaundice, however, are perhaps indicative of the latter, particularly inasmuch as they were observed in relatively early cases before one would anticipate significant hepatocellular functional impairment. Van den Bergh (23) was impressed by the coproporphyrinemia and increased coproporphyrinuria in mechanical jaundice, and ascribed them to regurgitation of bile into the blood. Gonzalez-Oddone, working in this laboratory, has observed⁵ that intravenously injected coproporphyrin appears in the thoracic duct lymph of dogs having ligated common bile ducts, in this respect behaving like injected bromsulfalein (24). Thus it appears that, in cases of jaundice, the coproporphyrin may appear in the urine in excess as a result either of hepatocellular or cholangiolar impairment, in the first instance probably being retained and in the second, regurgitated from the intrahepatic biliary system into the blood. The fact that the highest values are more likely to be encountered in jaundice due to hepatitis or cirrhosis may indicate a combination of these factors in such cases. This is quite in harmony with the belief that in hepatitis, there is both hepatocellular and cholangiolar injury, varying in severity in different instances and at different stages in the same instance (25).

It is appropriate to ask whether the increased

⁵ Unpublished observation.

amounts of coproporphyrin I in the urine of the present series of cases represents actual overproduction of this porphyrin, or merely a diversion of that which would normally be excreted in the bile and thence in the feces. There is actually no evidence of over-production. It has already been seen that the factors of retention and regurgitation are probably operative in reducing the biliary excretion of coproporphyrin and increasing its excretion in the urine. The amounts in the urine in these cases do not exceed those of the normal feces in 24 hours. Thus the evidence so far available indicates simple diversion rather than over-production, at least in the majority of cases in which type I is preponderant in the urine. This may be true as well for other conditions in which increased amounts of coproporphyrin I are found in the urine. Simultaneous studies of fecal and urinary excretion are needed, however, before the question can be answered with certainty. The available evidence at the present time (4, 5) indicates that there are but two conditions under which an overproduction of the type I isomer may be anticipated: (1) porphyria, a constitutional metabolic fault; (2) increased erythropoietic activity following increased blood destruction or blood loss. In the case of the type III isomer, it is clear that there is often marked overproduction and increased excretion from various causes (5).

It appears unlikely that a determination of the total urinary coproporphyrin will frequently be of value in the distinction of parenchymal jaundice from jaundice due to extrahepatic biliary obstruction. Careful study of the data obtained in the present study reveals considerable overlapping of values at various levels of icterus due to biliary obstruction as compared with diffuse liver injury. In Figure 4 the values for UCP in the cancerous, calculous, and hepatitis-cirrhosis groups are plotted against the values for total bilirubin. The data for the cases of cirrhosis included in this figure are considered in more detail in the subsequent paper of this series. It is seen in Figure 4 that while there is no distinct cleavage between the hepatitis-cirrhosis and the other groups, the higher values with lesser degrees of jaundice are more characteristic of the former. It appears from these data that values of 250 γ /24 hours, or higher, with total serum bilirubins less than 6.0 mg., as enclosed

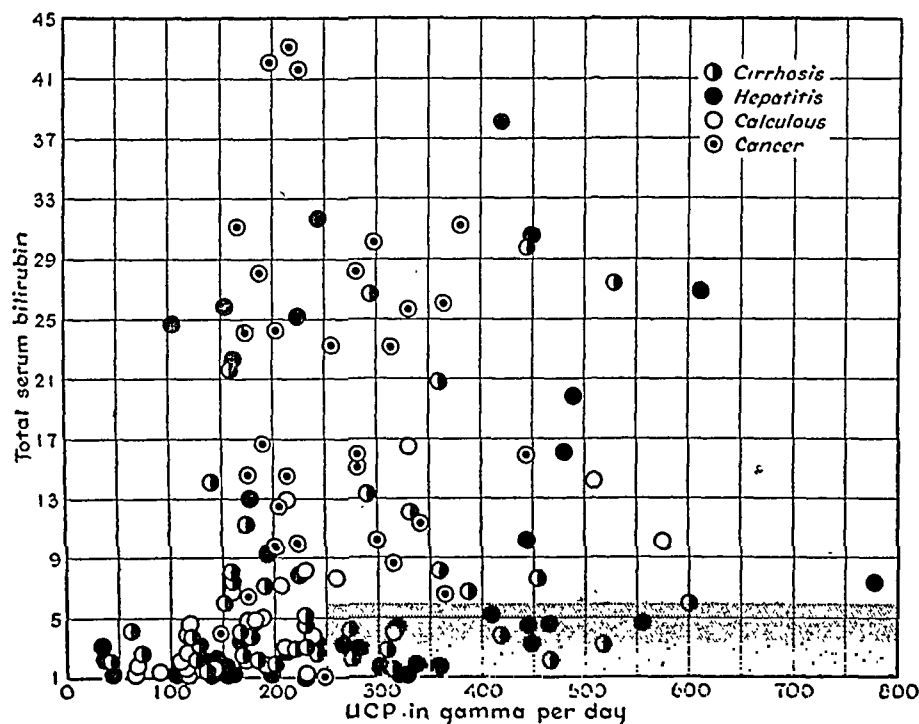


FIG. 4. UCP AND TOTAL SERUM BILIRUBIN IN CASES OF BILIARY TRACT CANCER, COMMON DUCT CALCULUS, INFECTIOUS HEPATITIS, AND CIRRHOSIS

in the shaded area in Figure 4, are much more likely to be associated with hepatitis or cirrhosis, than with extrahepatic biliary obstruction.

Insofar as hepatitis is concerned, the determination of the UCP is of more value in detecting residual hepatic functional impairment at varying intervals after the disappearance of jaundice. In the present series 45 of 95 cases exhibited elevated values at varying intervals after disappearance of jaundice.

In Table VIII, the cases for which data were given in Table II, are subdivided as to whether the

increase or lack of increase of the UCP was associated with other abnormalities, either symptomatic, physical or laboratory in character. It is seen that the UCP was elevated in 11 instances (groups 2a and 2c) in which the other laboratory findings were normal. Five of these had significant symptoms or physical findings and six did not. The largest number of cases were in group 2b in which there were symptoms or physical findings together with other abnormal laboratory data. It should be noted that in a number of instances in this group the other laboratory abnormalities

TABLE VIII

Groups with presence or absence of symptoms,* physical findings,* or abnormal laboratory data		Case nos. (as in Table II)	Total
1. Symptoms or physical findings without abnormal laboratory findings		3, 19, 29, 35, 37, 59	6
2. UCP > 100 γ /24 hr. (abnormal)			
a. Without symptoms or abnormal physical findings and without other laboratory abnormalities		4, 11, 15, 38, 49, 61	6
b. With symptoms or physical findings and with other laboratory abnormalities		5, 6, 7, 9, 11, 12, 14, 16, 17, 18, 21, 22, 23, 27, 28, 30, 32, 33, 34, 40, 42, 43, 44, 45, 46, 48, 49, 51, 52, 53, 54, 56, 57, 59, 60, 62	36
c. With symptoms or physical findings but without other abnormal laboratory data		8, 10, 26, 31, 36	5
d. With other abnormal laboratory data, but without symptoms or physical findings		39, 41	2
3. UCP < 100 γ /24 hr. (normal), with other laboratory abnormalities, and with or without physical findings		13, 20, 24, 47, 50, 55	6

* Symptoms > I, liver enl. > 1 cm., liver tenderness + or greater (see key, Table II).

were isolated; thus, in Cases 7, 9, 18, 21, 23, 34, and 59, the only abnormality in addition to the excessive UCP, was an elevated urine Ehrlich reaction; while in Cases 33, 36, 43 and 53 the only other abnormality was in the serum bilirubin level.

It was of particular interest to compare the UCP and the bromsulfalein retention, especially in those cases in which other findings were borderline. As noted in Table II, the bromsulfalein test was carried out 53 times in 43 cases. The UCP value corresponding in time with these determinations, was significantly elevated ($> 100 \gamma/24$ hours) in 22 instances in which the bromsulfalein retention did not exceed 5 per cent; conversely, there was but one instance in which the opposite was true. This was Case 13 in Table II, in which the bromsulfalein retention was 8 per cent and the UCP 92 $\gamma/24$ hours. Insofar as the UCP and cephalin flocculation tests are concerned, inspection of Table II reveals a negative flocculation in a number of instances in which the UCP was significantly increased; none are found in which the reverse was true.

At present there is no way of determining whether residual functional impairment is synonymous with persistent activity of a virus hepatitis.

From a practical standpoint, at any rate, it is probably wise to advise caution as to unusual activity, strain, fatigue, alcohol, etc., at least until the evidence of residual impairment has disappeared. Case 49 in Table II is an example of this problem. Further data for this case are given in Figure 2 and Table VII. This individual was permitted to resume his normal occupation in spite of persistent elevation of the UCP, but at the same time he was enjoined to avoid undue fatigue, irregular hours of sleep and alcohol. One of the problems of the recovery state of hepatitis has been that the individual may complain of persistent weakness and fatigue even though the results of various liver function tests are normal or borderline. In a number of such instances included in the present study, the elevated urinary coproporphyrin gave indication of residual activity or impairment, and appeared to be correlated with the symptoms of fatigue, weakness or nervousness. Conversely, however, we have observed several individuals complaining of one or more of these symptoms yet having normal porphyrin values in addition to normal composite liver function studies. In such instances it has been impossible to decide with certainty whether the symptoms were on a psychic

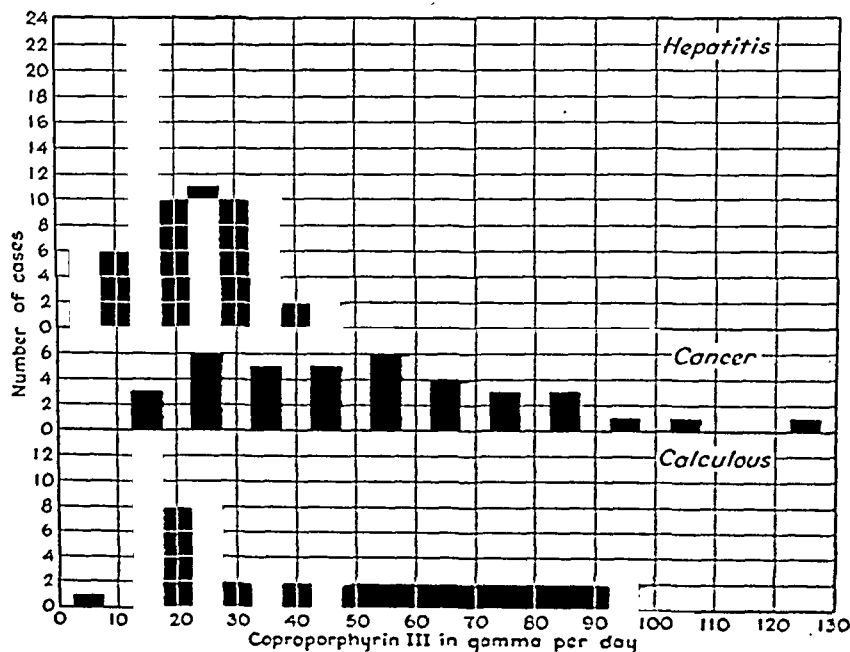


FIG. 5. FREQUENCY DISTRIBUTION OF VALUES FOR URINARY COPROPORPHYRIN III IN CASES OF HEPATITIS, BILIARY TRACT CANCER, AND COMMON DUCT CALCULUS

basis or were in reality due to residual hepatic impairment. Additional studies, including exercise tolerance with relation to coproporphyrin excretion, are desirable in order to determine the degree of assistance one may expect from this determination.

From the present results it is evident that the increase in UCP in hepatitis and mechanical jaundice is due in the main to increased excretion of the type I isomer. Inspection of the data in Tables III, IV, V and VI, reveals, however, that in some instances the amount of type III isomer exceeds the normal upper limit of 34 γ /24 hours reported previously (14). In the hepatitis and infectious mononucleosis groups there are few of these and the excess is slight, probably within the limit of error of the differential precipitation method (12, 13). The two outstanding exceptions were cases in the calculous group which have already been referred to; one of these had a necrotic abscess with marked splenomegaly, and the other had miliary tuberculosis. In one additional instance (Case 13 in Table VI) an unexplained absolute increase of type III coproporphyrin is noted, comprising, however, but 17–18 per cent of the total UCP. In the cancer group, there are quite a number of cases having considerable excesses of type III isomer even though type I is clearly preponderant in each instance. Frequency distribution curves for the three groups are shown in Figure 5. Several cases, already discussed, have been omitted in the preparation of this figure, because of significant peculiarities believed to place them in separate categories. These cases are numbers 51 in Table II, 2 and 12 in Table VI. Figure 5 reveals the relative frequency of increased coproporphyrin III excretion in the cancer group. Nineteen of 39 cases in this group had values above 50 μ g. in 24 hours, while all of the 40 in the hepatitis group were below this level. Thus it is evident that the patients with cancer of the biliary tract or liver vary significantly in the composite, from the other groups, and especially from the hepatitis group, in respect to porphyrin metabolism. It appears that an absolute increase of the type III isomer greater than 50 γ /24-hour urine sample, favors the diagnosis of cancer rather than hepatitis, and is much more likely to be encountered with cancer than with calculus, in the presence of regurgitation jaundice. The reason for this difference is not

clear, but the possibility may be entertained of an endogenous chemical intoxication, from necrotic or abnormal cells. It is recognized too, that a constitutional factor cannot be excluded.

SUMMARY AND CONCLUSIONS

1. The total urinary coproporphyrin (UCP) is regularly increased above the upper limit of normal of 100 γ /24-hour urine sample, both in infectious hepatitis during the period of jaundice, and in cases of mechanical jaundice. The increase in cases of hepatitis often persists in some measure for considerable periods after the disappearance of jaundice and after the return to the normal range of conventional tests such as bromsulphalein retention and cephalin cholesterol flocculation. In certain instances the increase of the UCP has constituted the only objective evidence of residual hepatic functional impairment.

2. In cases of infectious mononucleosis the UCP is commonly increased, usually in association with other evidence of hepatic functional impairment.

3. Isomer analysis has shown that the increase in UCP in infectious hepatitis, infectious mononucleosis, and mechanical jaundice, is mainly due to the type I isomer. Certain exceptions are discussed and it is shown that in cases of cancer of the pancreas, bile ducts, or liver, the increased UCP, although preponderantly type I, includes an absolute increase of type III with relative frequency. This has not been observed to any significant degree in cases of infectious hepatitis, infectious mononucleosis, or uncomplicated calculous jaundice.

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STUDIES ON VASOMOTOR TONE. I. THE EFFECT OF THE TETRAETHYLAMMONIUM ION ON THE PERIPHERAL BLOOD FLOW OF NORMAL SUBJECTS^{1, 2}

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The animal experiments of Acheson, Moe, and Pereira have shown that the tetraethylammonium ion is capable of relatively complete blockade of sympathetic and parasympathetic impulses at the autonomic ganglia (1-2). In the dog, the infusion of 20 mg. per kg. per hour will completely prevent the cardiac response to vagal stimulation, and the response of the nictitating membrane to preganglionic stimulation of the cervical sympathetic nerve (3). That the drug would also inhibit autonomic tone in man has been demonstrated in previous reports (4-7).

The purpose of the present investigation was 1. to study the effect of tetraethylammonium on blood flow in the extremities of normal and sympathectomized persons by the more quantitative plethysmographic technique; 2. to establish the extent of sympathetic vasomotor blockade produced by the drug; and 3. to compare its effect on peripheral blood flow with that of certain other vasodilating agents in common use.

METHODS

Peripheral blood flow was measured by means of a venous occlusion plethysmograph⁴ according to the technique described by Abramson (8). Collecting cuff pressures about 20-30 mm. of mercury below diastolic levels were selected and all subsequent determinations in any experiment were made at the same pressure in the collecting cuff.

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⁴ We are indebted to Dr. I. Arthur Mirsky for the loan of the plethysmographs used in this investigation.

All blood flows were determined with the subject in the recumbent position, the room temperature at approximately 70° F and bath temperature at 32° C. Foot blood flow was determined with the subject horizontal and the plethysmograph at body level. Forearm and calf blood flows were determined after excluding return circulation from the hand and foot by means of tourniquets inflated to exceed arterial pressure for five minute periods during which consecutive blood flow measurements were made.

After the subject had rested 45 to 60 minutes, three to four series of blood flow determinations were performed. Each series consisted of five observations made at 30 second intervals in the resting state. During the experimental procedure blood flow was determined every 30 seconds for 10 to 15 minutes or until a maximum had apparently been reached. The average of at least five consecutive determinations just prior to the test procedure was designated as the *average resting blood flow* and of five consecutive determinations at the time of maximum increase as the *average maximal blood flow*.

In the sympathetic block experiments, resting blood flows were first obtained with the foot in the plethysmograph. It was then removed from the apparatus and 0.5 per cent metycaïne infiltrated in the region of L₁, L₂, and L₃ on one side according to the technique described by White and Smithwick (9), until paravertebral block had begun to take effect as judged by an increase in toe temperature with abolition of the temperature gradient between toe and thigh. The foot was then replaced as rapidly as possible in the plethysmograph and observations were repeated at frequent intervals until blood flow had fallen toward resting levels. That we were able to record the maximum flow after paravertebral block seemed apparent from the fact that the blood flow continued to increase for a while after the foot had been replaced in the plethysmograph and no decline appeared until after 15 to 30 minutes of observations.

Skin temperatures in the extremity opposite to the one enclosed in the plethysmograph were recorded by means of a Leeds-Northrup potentiometer, the thermocouples being held lightly in contact with the skin on the outside margin of the tip of the big toe or index finger, and on the anterior surface of the upper arm or thigh.

The normal subjects referred to in this report consisted of seven healthy male college students in whom the effects of varying dosage of tetraethylammonium,

other vasodilating procedures, and paravertebral block were compared. The remaining normal subjects consisted of 16 male and seven female patients ranging in age from 18 to 43, hospitalized for a variety of disorders but presenting no evidence suggesting the presence of any type of vascular disease.

OBSERVATIONS

1. Effect of tetraethylammonium on peripheral blood flow in normal subjects

In Table I are presented the effects of the intravenous injection of 500 mg. of tetraethylam-

TABLE I
Effect of tetraethylammonium chloride on peripheral blood flow and skin temperature in normal subjects

	Subject	Sex	Age	Blood flow* cc./100 cc./limb/min.		Ratio TEA to resting flow	Mean B.P. per cent change after TEA	Skin temperature gradient† ° C			
				Before TEA	After TEA			Toe-thigh		Finger-arm	
								Before TEA	After TEA	Before TEA	After TEA
Foot	H. T.	M	21	4.2	12.0	2.9	11	1.8	-2.0		
	W. P.	M	18	1.1	9.2	8.3	-12	5.6	-2.8		
	O. O.	M	31	2.9	8.1	2.8	-10	4.0	0.3		
	G. M.	M	25	1.4	6.5	4.7	- 8	4.7	-0.3		
	E. M.	M	21	2.0	6.2	3.1	0	9.0	0.6		
	D. M.	M	24	1.2	12.5	10.3	4	6.0	-2.0		
	T. G.	M	36	2.1	9.8	4.7	- 8	3.3	-2.0		
	B. F.	M	43	0.8	5.3	6.6	-10	7.0	2.0		
	R. E.	M	18	2.0	9.6	4.8	5	7.2	-0.4		
	J. B.	M	37	3.8	8.5	2.2	- 3	9.0	0.9		
	J. D.	M	21	4.4	15.0	3.4	2	2.8	-2.2		
	D. S.	F	27	1.1	6.4	5.8	-13	8.0	-0.5		
	B. M.	F	21	1.1	16.0	14.5	+ 8	6.8	-3.6		
	M. R.	F	19	0.5	14.9	33.0	+20	7.0	1.6		
Average				2.04	10.0	7.65					
Hand	J. S.	M	37	3.1	3.8	1.2	-10			7.0	7.0
	E. W.	M	33	4.9	14.6	3.0	4			2.0	1.2
	W. R.	M	36	2.2	8.1	3.7	- 7				
	J. M.	M	30	5.0	26.3	5.2	17				
	R. F.	M	19	5.2	19.2	3.7	-17				
	H. E.	M	18	5.3	23.9	4.5	- 2				
	J. M.	F	21	1.2	10.7	9.0	9			2.7	-0.3
M. A.	F	27	1.6	12.5	7.8	3			9.0	6.0	
Average				3.5	14.8	4.8					
Forearm	J. D.	M	21	2.6	5.0	1.9	2			1.5	1.7
	H. E.	M	18	1.9	4.9	2.6	1			5.4	-1.0
	D. M.	M	24	3.0	4.8	1.6	15			8.1	0
	E. M.	M	21	1.0	1.4	1.4	-20			1.0	1.0
	G. M.	M	26	2.0	1.8	0.9	- 3			3.0	3.0
	H. T.	M	21	1.2	2.0	1.7	0			4.5	0
	R. S.	F	22	2.1	5.2	2.5	2			4.2	0
	M. B.	F	21	2.8	3.2	1.1	0			7.9	0
Average				2.07	3.53	1.7					
Calf	L. S.	M	18	0.7	1.1	1.6	- 7				
	A. H.	M	31	1.3	1.4	1.1	3				
	D. H.	M	16	0.5	1.2	2.4	0				
	D. W.	F	24	0.5	0.8	1.6	10				
	C. M.	F	15	0.8	1.2	1.5	5				
	C. B.	F	25	0.3	0.5	1.7	4				
Average				0.7	1.0	1.6					

500 mg. of tetraethylammonium chloride were given intravenously in all experiments.

* Blood flow values were calculated as described in the text. The value before TEA represents the average resting flow and that after TEA was the average maximal flow.

† Skin temperatures were determined on the extremity opposite to the one enclosed in the plethysmograph. Arm or thigh temperatures were not significantly altered by injection of TEA; consequently changes in gradient represent increases in toe or finger temperatures. Negative values indicate that digital temperatures exceeded skin temperatures of arm or thigh.

EFFECT OF INTRAVENOUS INJECTION OF TE-A-CL ON BLOOD FLOW IN FOOT

H.T. NORMAL 21 YR. OLD W. ♂

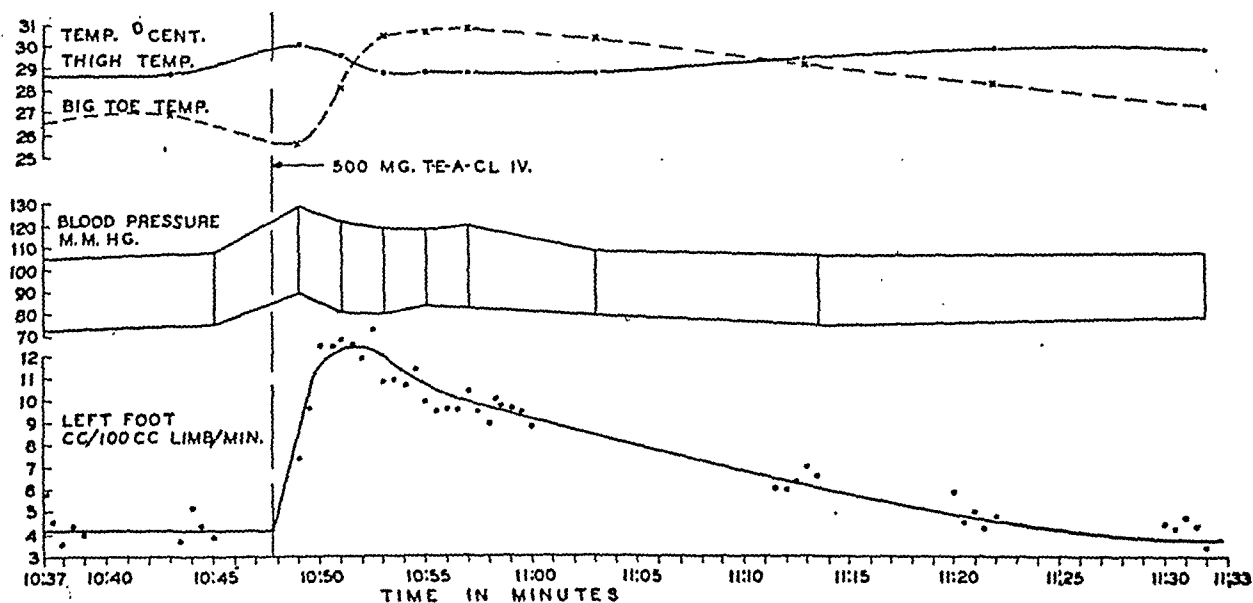


FIG. 1. TYPICAL RESPONSE OF NORMAL SUBJECT TO INTRAVENOUS INJECTION OF 500 MG. OF TETRAETHYLAMMONIUM CHLORIDE

monium chloride⁵ on peripheral blood flow and skin temperature. After the rapid intravenous injection of the drug, the blood flow in the foot always increased; that in the hands usually rose; while calf and forearm blood flows were unchanged or increased only slightly. In the few female sub-

jects studied there was a tendency for the drug to increase the blood flow in the foot and hand, more than in normal male subjects. The skin temperature gradient between toe and thigh was always reduced and often abolished while the temperature gradient between finger and forearm was less regularly affected. A typical response is shown in Figure 1. Foot volume usually increased after the administration of the drug and reached its

⁵ We are indebted to the Department of Clinical Investigation, Parke, Davis & Co., for the supply of "Etamon" used in these studies.

EFFECT OF INCREASING DOSES OF TEA ON BLOOD FLOW OF LEFT FOOT AS DETERMINED BY VENOUS OCCLUSION PLETHYSMOGRAPH

35 E. MC. NORMAL SUBJECT

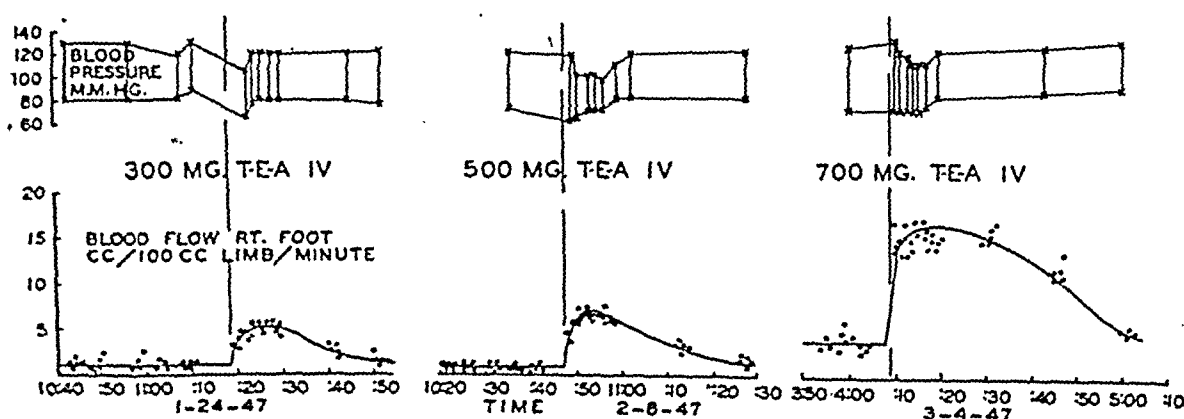


FIG. 2. INCREASE IN BLOOD FLOW IN THE FOOT AFTER TETRAETHYLAMMONIUM IS APPROXIMATELY PROPORTIONAL TO THE DOSE ADMINISTERED

peak at the time of maximum flow. The systemic blood pressure did not fall markedly in any of the normotensive subjects studied, but pulse rate increased moderately after the drug. It was shown that tachycardia per se apparently had no effect on the plethysmographic measurement since comparable increases in pulse rate induced by the administration of atropine, 1 mg. intravenously to three normal subjects, had no significant effect on the foot blood flow.

2. Effects of dosage and route of administration

In three normal subjects it was demonstrated that greater and more prolonged increases in foot blood flow were obtained when 500 mg. rather than 300 mg. of the drug were given intravenously. A 500 mg. dose was not exceeded except in one

case in which 700 mg. produced a greater rise in blood flow than 500 mg. (Figure 2). In one subject the intramuscular injection of 20 mg. per kg. resulted in a more prolonged but no greater rise in blood flow than a 500 mg. dose given intravenously.

3. Effect of tetraethylammonium on reflex vaso-motor changes

Following the injection of tetraethylammonium, the reflex vasoconstrictor response to pinprick and mental arithmetic was reduced or absent. Respiratory and other rhythmic changes in limb volume were reduced (Figure 3). Reflex vasoconstriction induced by suddenly immersing an opposite extremity in ice water was likewise obliterated by tetraethylammonium (Figure 4).

REFLEX CHANGES IN LIMB VOLUME BEFORE AND AFTER TEACI

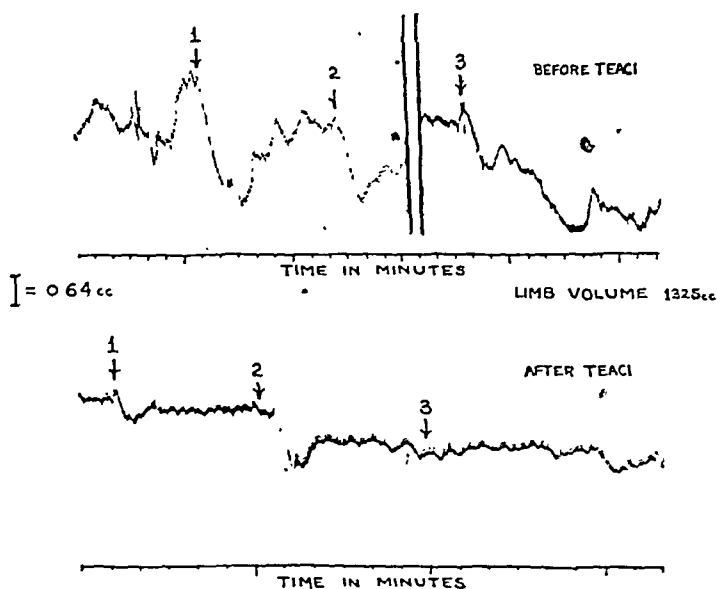


FIG. 3. REDUCTION IN REFLEX CHANGES IN LIMB VOLUME AFTER AUTONOMIC BLOCKADE WITH TETRAETHYLAMMONIUM

The upper tracing is a control plethysmographic observation. The lower record was obtained eight minutes after the administration of 500 mg. of tetraethylammonium chloride intravenously. At 1 and 2 a painful stimulus (pin prick) was applied. At 3 the patient was given a problem in mental arithmetic to perform.

Note the reduction in rhythmic volume changes and in the response to various stimuli after autonomic block.

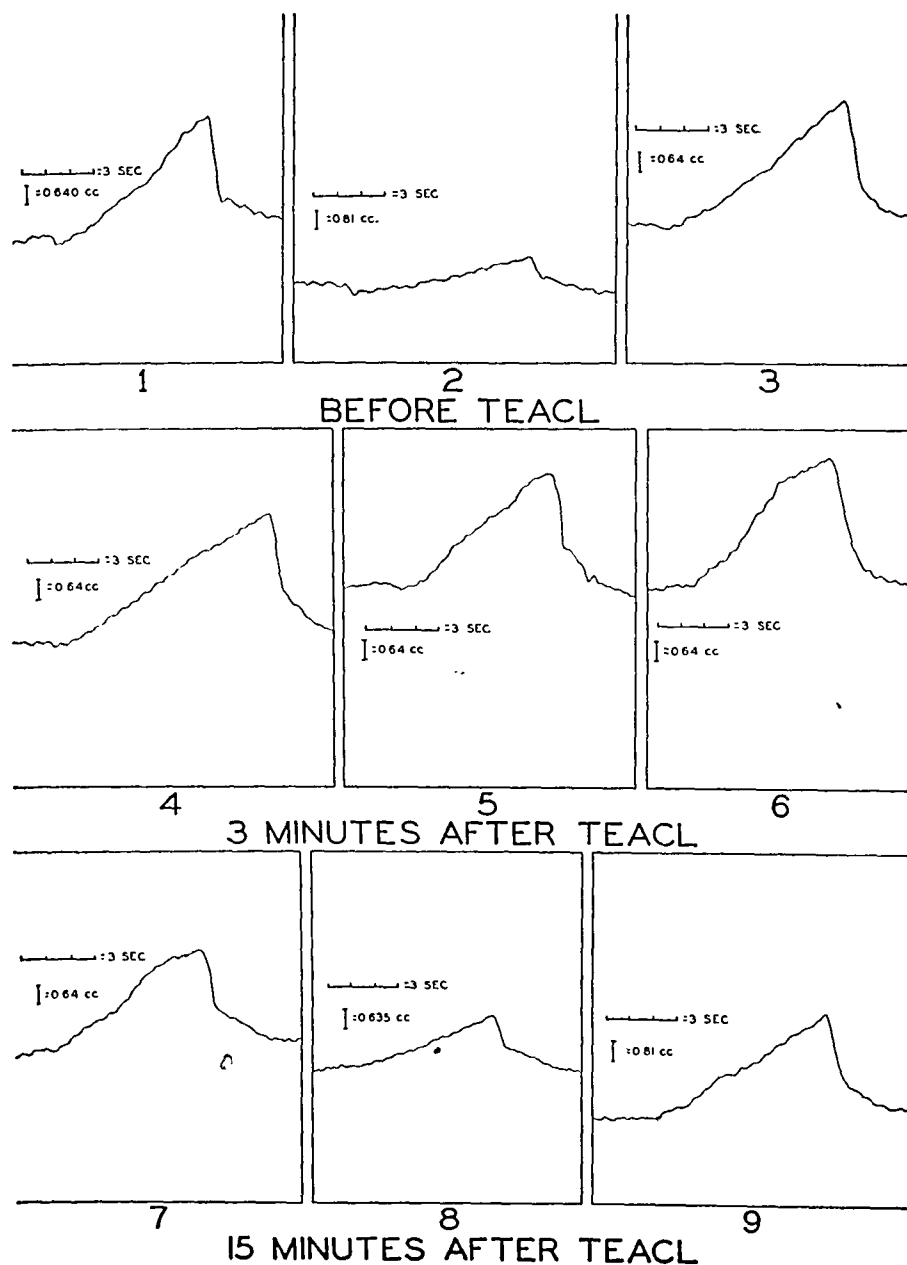


FIG. 4. BLOCKADE OF REFLEX VASOCONSTRICTION TO COLD STIMULUS AFTER AMMONIUM

Representative inflow curves in the hand have been selected from the plethysmographic tracings of a normal subject before and after 500 mg. of tetraethylammonium chloride intravenously. Numbers 2, 5, and 8 were recorded 60 seconds after immersion of the opposite hand in ice water. The tracings on the left and right represent inflow curves one minute before and $1\frac{1}{2}$ minutes after cessation of the ice stimulus.

It will be noted that the reflex vasoconstriction apparent in the first series of tracings was abolished three minutes after tetraethylammonium chloride and had begun to return 15 minutes after the injection.

1. Effect of tetraethylammonium compared to that of other vasodilating procedures

(a) Heat

In seven normal subjects vasomotor tone was reduced by heating the body and three extremities under an electric blanket for 30 to 45 minutes until

marked diaphoresis occurred and no further increases in blood flow in the foot could be produced. The increases obtained by this procedure showed considerably greater variation and were somewhat less than after a single injection of 500 mg. of tetraethylammonium (Figure 5). When the drug

was given at the point of maximum reflex vasodilatation, only minimal increases in blood flow were observed.

(b) Vasodilating drugs

Observations on the effect of nicotinic acid, papaverine, aminophyllin, and nitroglycerin on foot blood flow were made in several normal male subjects in whom the effects of tetraethylammonium had also been studied. These drugs had far less effect than did tetraethylammonium given to the same subjects (Figure 5).

(c) Lumbar paravertebral block

In four instances lumbar paravertebral block was induced with metycaine before or after the response of the vascular bed in the extremity to tetraethylammonium had been tested. The block was usually slightly more than twice as effective as

tetraethylammonium in increasing blood flow to the foot, and the hyperemia lasted from three to four times as long (Figures 5, 6). In cases where block appeared incomplete, as judged by a delayed or incomplete rise in toe temperature before placing the foot in the plethysmograph, and by a less than usual increase in blood flow, injection of tetraethylammonium resulted in further vasodilatation. Also when sympathetic block had begun to wear off, as manifested by a decline in blood flow in the foot, an increase in blood flow could be obtained by an injection of tetraethylammonium.

5. Effect of tetraethylammonium on the sympathectomized extremity

In one patient with early Buerger's Disease, tetraethylammonium increased blood flow in the foot from 3 cc. to 10 cc. per 100 cc. of limb volume per minute. After lumbar sympathectomy blood

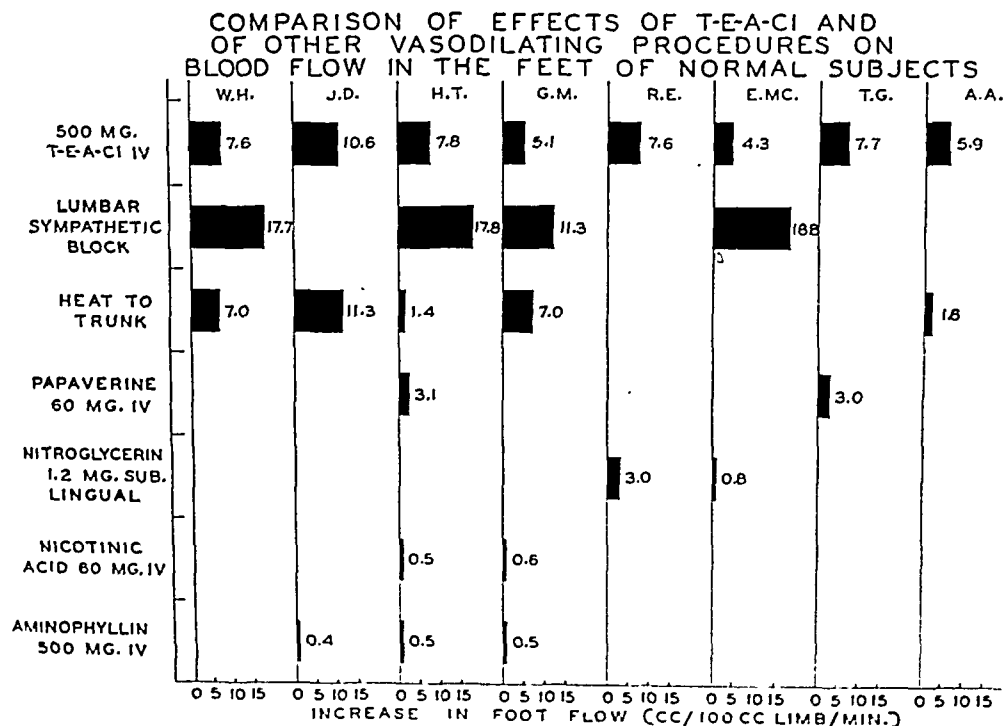


FIG. 5

Length of the solid bars indicates the increase in blood flow after the various procedures described in the left-hand margin of the figure. The increase in foot blood flow in centimeters per 100 cc. foot volume per minute is expressed numerically beside each bar. Experiments performed in the same individual on different days are arranged in vertical columns beneath the subject's initials.

It will be observed that paravertebral block was the most effective vasodilating procedure, that tetraethylammonium chloride and reflex body heating produced about one-half as great an increase in foot blood flow and that certain common vasodilator drugs were considerably less effective.

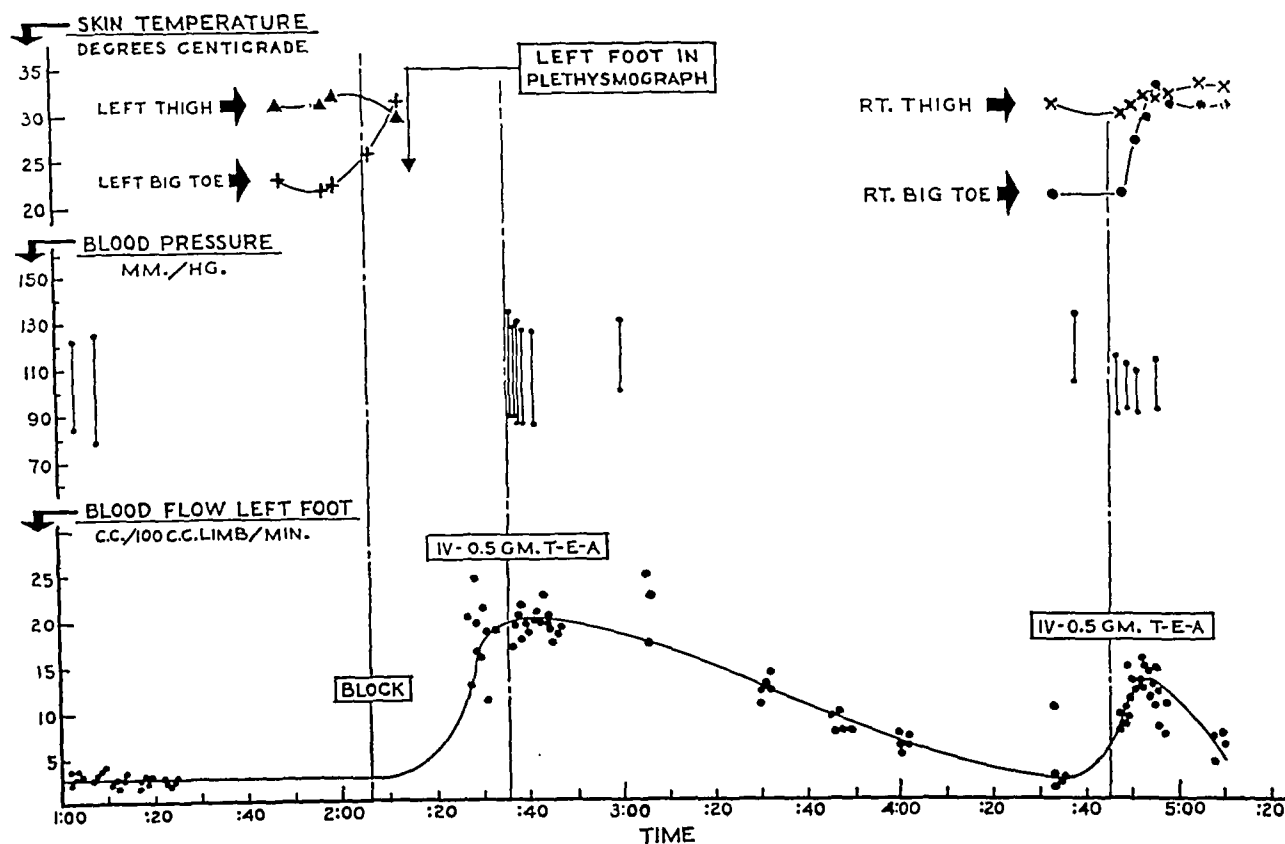


FIG. 6. COMPARATIVE EFFECT OF TETRAETHYLAMMONIUM AND OF LUMBAR PARAVERTEBRAL BLOCK WITH METYCAIN ON FOOT BLOOD FLOW IN NORMAL SUBJECT

Note that at height of sympathetic block administration of tetraethylammonium produced no significant change in blood flow but that after the effects of the metycain had worn off, tetraethylammonium produced vasodilatation of somewhat lesser magnitude and duration.

flow averaged 5 cc. per minute but injection of the drug was now without vasodilator effect (Figure 7). On 12 other occasions we have observed that sympathectomy has abolished the vasodilator response to tetraethylammonium. These observations have been made four times with respect to blood flow in the hand and eight times with respect to blood flow in the foot, and as early as the second day after operation and as late as the ninth month following sympathectomy.

DISCUSSION

Tetraethylammonium significantly increased blood flow to the foot in every normal subject studied; it produced vasodilatation in the hand in seven of eight instances; while circulation in the forearm and calf increased only slightly in most of the subjects. These plethysmographic observations correspond to those of Slaughter, Brown, and Wakim (10) who used an air displacement plethysmograph and measured circulatory changes in the hand and forearm or foot and calf.

The increases in temperature following tetraethylammonium provide confirmatory evidence of the peripheral vasodilator action of the drug. This occurred regularly in the toes and frequently in the fingers. Elevation in skin temperature in the extremities of subjects with vasospastic peripheral vascular disease has been reported previously (4-7). On the other hand, Boyd *et al.* (11), and DeBakey *et al.* (12) have found elevations of the digital temperature to occur less consistently both in the normal and in the subject with peripheral vascular disease. We are unable to explain these discrepancies but we believe that the plethysmographic observations herewith reported provide convincing evidence of the vasodilator action of the drug, at least so far as the blood flow in the entire hand or foot are concerned.

Since tetraethylammonium no longer produces vasodilatation after an extremity has been sympathectomized, it follows that the drug acts by altering sympathetic tone and not by any direct vasodilator action on the blood vessels. This conclusion

is further supported by the observations that the vasodilator response is reduced or abolished when vasoconstrictor tone is reduced by body heating and by the observation that vasoconstrictor reflexes following pain, cold, and mental activity are reduced or abolished during the period of action of the drug. Finally, the less marked increases in blood flow in the forearm or calf following tetraethylammonium can be readily explained by the relative lack of vasomotor tone to these vascular areas (8). Our clinical findings, therefore, are not at variance with the results of investigations performed in animals (1, 2). While it is impossible to prove the ganglionic site of action in the human subject as in the animal, it seems most likely that blockade of sympathetic impulses occurs in this location.

If it may be assumed from our studies that lumbar paravertebral block produced complete inhibition of vasomotor tone in the foot, the maximum blood flow under these conditions averaged 17.6 cc. per 100 cc. of foot volume in four normal

subjects. This figure may be compared to an average maximum flow of 9.9 cc. after administration of 500 mg. of tetraethylammonium. It would appear that the increase in blood flow after this drug represented about one-half the possible increase had a complete chemical block been established. In larger doses it is probable that a more complete blockade could have been achieved since in one subject the response to the injection of 700 mg. of the drug was greater than after the administration of 500 mg. and approximated the degree of vasodilatation to be expected from complete sympathetic block. However, we do not believe that in the average human subject more than 500 mg. can be administered with safety by the intravenous route. The failure to achieve greater inhibition of sympathetic tone was somewhat unexpected since the autonomic nervous system of the dog can apparently be blocked almost completely to pre-ganglionic faradic stimulation for a brief interval by doses of 4 to 8 mg. per kg. (3). If this were applied to a human subject weighing 60 kg., it would

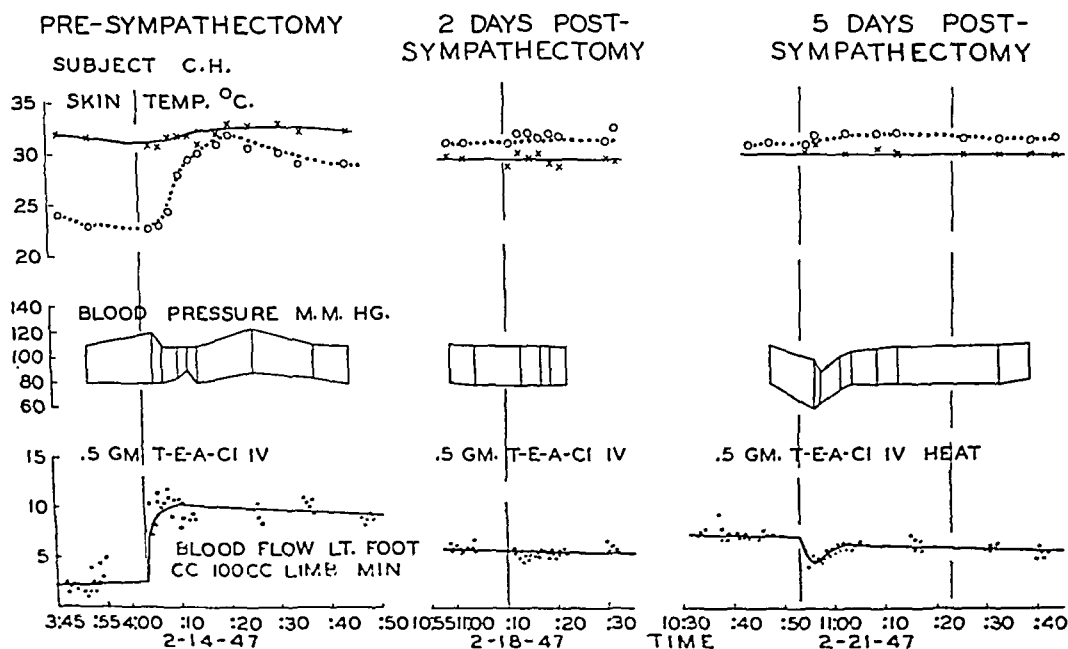


FIG. 7. EFFECT OF TETRAETHYLAMMONIUM ON BLOOD FLOW BEFORE AND AFTER SYPHATHECTOMY

Vasodilator response to tetraethylammonium occurring prior to sympathectomy in a patient with early Buerger's Disease was abolished by the operation. Failure to increase blood flow in sympathectomized extremity indicates that the drug has no direct vasodilator action in this dosage.

The slight decline in blood flow after tetraethylammonium on fifth post-operative day was probably the result of parallel decline in blood pressure. It will be noted that body heating likewise failed to produce vasodilatation on the fifth day following sympathectomy.

mean that 480 mg. given intravenously should have produced a temporary but relatively complete blockade.

Gibbon and Landis (13) and Collier and Maddock (14) have utilized reflex heat vasodilatation as a means of inhibiting sympathetic vasomotor tone. The degree of vasodilatation achieved was measured by alterations in the digital temperature. The extent of the peripheral vasodilatation, as measured in the plethysmograph, which could be induced by prolonged body heating in five normal subjects was more variable and of a lesser magnitude than the response to 500 mg. of tetraethylammonium. Despite the frequent obliteration of the temperature gradient between toe and thigh following both these procedures, the increases in blood flow were far less than after paravertebral sympathetic block. It would appear from these observations that reflex heat vasodilatation likewise does not produce a complete release of vasomotor tone and the abolition of a skin temperature gradient does not necessarily mean that a complete sympathetic blockade has been achieved.

While we made no extensive comparative studies of the effects of various vasodilator drugs on blood flow in the foot, a few observations utilizing the plethysmographic technique have convinced us that, at least in the normal subject, measures involving an inhibition in vasoconstrictor tone result in significantly greater increases in blood flow than do those vasodilating agents which act on the peripheral vessels directly. The increase in blood flow following papaverine, nitrites, nicotinic acid, and aminophyllin given intravenously was far less than that seen after lumbar sympathetic block, tetraethylammonium, or reflex heat vasodilatation. The extensive studies of Abramson and his associates (8) on the effect of various vasodilator drugs and of Gubner *et al.* (15) on the effects of glycine would suggest that increases in the peripheral blood flow of the magnitude which followed tetraethylammonium are most uncommon.

CONCLUSIONS

1. Tetraethylammonium in doses of 500 mg. intravenously produces an average seven-fold increase in foot blood flow, a four-fold increase in hand blood flow, and slight increases in forearm

and calf blood flows in the normal subject as measured by the venous occlusion plethysmograph. Digital skin temperatures are increased and the temperature gradient between thigh and toe and forearm and finger are usually abolished or greatly reduced.

2. The drug does not produce vasodilatation in the sympathectomized extremity. Its vasodilator action is therefore the result of the inhibition of sympathetic vasoconstrictor tone.

3. Sympathetic blockade is probably incomplete with the usual doses of tetraethylammonium tolerated by the human subject. The vasodilatation in the foot, which follows 500 mg. of the drug, is about 50 per cent of that observed after lumbar paravertebral block.

4. In the foot of the normal subject, the increase in blood flow is of much greater magnitude after tetraethylammonium than after aminophyllin, papaverine, nicotinic acid, and nitroglycerin and is slightly greater than the vasodilatation following prolonged body heating.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Mrs. Richard Tybout, Mrs. Charles Sempere, Mrs. Fred Shelkun, and Miss Celena Axelrod for their technical assistance in these studies.

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THE EFFECT OF ANOXIC ANOXIA ON THE HUMAN KIDNEY¹

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Congestive heart failure is associated with reduction of renal blood flow and retention of salt and water. The possibility has been raised that these changes may result from the concomitant renal anoxia (1, 2). The present studies are concerned with the effect of anoxia on the human kidney under situations of normal cardiovascular dynamics.

METHODS

In subjects with normal cardiovascular and respiratory systems, an arterial blood oxygen tension of approximately 50 mm. Hg was induced by the inhalation of 14 per cent oxygen. This concentration of oxygen was selected in order to induce a maximum degree of anoxia compatible with minimal changes in cardiovascular and respiratory dynamics (3). Observations of discrete renal functions were then made in these subjects and compared with renal functions while breathing room air. Observations were also conducted on emphysematous individuals who had arterial blood oxygen tensions of approximately 50 mm. Hg while breathing room air and observations were repeated while breathing 100 per cent oxygen. In the latter cases, periods while breathing room air were considered to be anoxic periods and periods while breathing 100 per cent oxygen were considered control periods. In all cases, 20 to 45 minutes were allowed for equilibration following a change of oxygen concentration in respired air.

Glomerular filtration rate was measured as the inulin clearance and renal plasma flow as *p*-aminohippurate clearance, following the principles outlined by Smith (4). An intravenous infusion of saline containing inulin and sodium *p*-aminohippurate was administered at a rate of 3 ml./min. subsequent to an appropriate priming dose. Urine was collected in periods of 15 to 30 minutes through an indwelling multi-eyed catheter. At the end of each period the bladder was rinsed with distilled water. Urine samples for measurement of pH were collected and kept under oil. At the mid-point of each urine collection period, blood was drawn anaerobically from the femoral

artery through an indwelling needle. Heparin was used as an anticoagulant. The renal data in the tables represent the means of three collection periods.

Since the inulin and *p*-aminohippurate were administered in an infusion, the addition of a small amount of normal saline to the circulating volume (500 ml. over 3 hours) might be responsible for the observed changes in electrolyte excretion. To obviate this factor, the time relationship of the control to anoxic periods was varied. In CH, LS, LB, MH, and MS, the control periods preceded the anoxic periods. In KN, DV, and NG, the control periods followed the anoxic periods. MS was further studied to follow his return to the control levels.

Inulin was measured in urine and plasma by Harrison's modification (5) of Alving's method (6). *p*-Aminohippurate was measured in diluted urine and trichloroacetic acid filtrates of plasma by the Bratton-Marshall reaction (7). Sodium and potassium were determined with an internal standard flame photometer (8). Chloride was determined according to Wilson and Ball (9); phosphorus according to Fiske and SubbaRow (10); ammonia according to Summerson's modification of the Van Slyke and Cullen procedure (11); pH with a Leeds and Northrup pH meter; urinary protein was determined by the micro-Kjeldahl technique using a trichloroacetic acid precipitation.

Expired air was collected in a Tissot spirometer which recorded respiratory rate and volume. The mouth piece and nose clip were kept on the subject throughout the procedure except for a few minutes rest prior to a change in oxygen concentration in the inspired air. Arterial blood oxygen and carbon dioxide contents were determined by the method of Van Slyke and Neill (12), and tensions by the direct method of Riley (13). Respiratory gases were analyzed in a Haldane-Henderson apparatus and micro-Scholander gas analyzer (14).

The pulse rate and brachio-arterial blood pressure were recorded at five-minute intervals throughout any particular procedure. Mean arterial blood pressure levels were calculated by adding a third of the systolic and diastolic difference to the diastolic pressure.

With a view to minimizing the problem of anxiety, prior to the day of experiment the subjects were catheterized and were acclimated to breathing through a Tissot spirometer. The subject was not informed of changes in oxygen concentration during the experiment and gave no indication of having noticed the change.

¹ This investigation has been aided by grants from the Josiah Macy Jr. Foundation and the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

OBSERVATIONS

There were slight alterations in respiration and circulation during inhalation of 14 and 100 per cent oxygen (Table I). The changes in respiration were all within the error of measurement. The average pulse rate did not change significantly in four observations while it rose 8 to 14 beats during anoxia in the other five. The average mean blood pressure during the anoxic periods was not different from that of the control except in one instance when it rose 13 mm. Hg during anoxia.

Renal plasma flow did not decrease during the anoxic periods. On the contrary, there was a tendency for it to increase. In observations in individuals with normal pulmonary function, three comparisons exhibited no appreciable change in renal plasma flow and four increased 13 to 20 per cent. In the two subjects with pulmonary emphysema, the administration of 100 per cent oxygen induced a fall in renal plasma flow; considering periods of breathing room air as the anoxic periods there was an increase in renal plasma flow of 29 per cent in each subject during anoxia. The filtration rate did not change appreciably during anoxia in four observations, increased 9 to 23 per cent in four, and decreased 19 per cent in one.

The interpretation of *p*-aminohippurate clearance as a measure of renal plasma flow is based on its almost complete extraction by the kidney. Under normal situations this extraction is at a maximum. A decrease in *p*-aminohippurate clear-

ance during anoxia would be difficult to interpret in terms of renal plasma flow if the anoxia altered its extraction. However, the observed clearances increased and a change in this direction can be interpreted as an increase in effective renal plasma flow.

In the five comparisons where a change in pulse rate did occur, those which evidenced the greatest change in pulse rate did not have the greatest change in renal plasma flow. Since it has been observed that the increase in cardiac output which occurs during inhalation of anoxic gas mixtures results from an increase in pulse rate rather than stroke volume (15, 16), the observed changes in renal plasma flow are not considered to reflect changes in cardiac output.

There was an invariable and frequently marked increase in the rate of sodium and chloride excretion during anoxia (Table II). Sodium increased from a mean excretion of 162 μ Eq./min. to 256 μ Eq./min. Chloride increased from a mean excretion of 161 μ Eq./min. to 245 μ Eq./min. The urine flow tended to increase during anoxia presumably in consequence of the increased concentration of sodium and chloride in the urine. The magnitude of change in salt and water excretion exhibited little if any correlation with the magnitude of change in filtration rate or renal plasma flow.

Other electrolyte changes which occurred during anoxia were not as marked as those of sodium and

TABLE I
Effect of anoxia on respiratory, cardio-vascular, and renal dynamics

Subject	Pulmonary disease	Arterial blood oxygen tension		Respiratory minute volume			Pulse rate*			Mean arterial blood pressure*			Inulin clearance†			<i>p</i> -Aminohippurate clearance†		
		Control	Anoxia	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change
		mm. Hg	mm. Hg	Lits/min.	Lits/min.	Lits/min.	Beats/min.	Beats/min.	Beats/min.	mm. Hg	mm. Hg	mm. Hg	ml./min.	ml./min.	per cent	ml./min.	ml./min.	per cent
CH†	None	97.6	54.3	4.7	5.3	+0.6	79	82	+3	89	88	-1	95	90	-5.3	394	389	-1.3
LS†	None	99.8	50.1	8.1	7.3	-0.8	82	84	+2	106	103	-3	111	121	+9.0	601	682	+13.5
LB†	None	97.0	61.4	7.4	8.7	+1.3	59	62	+3	95	96	+1	101	82	-18.8	613	546	-10.9
MH†	None	96.9	49.0	7.3	8.9	+1.6	67	75	+8	100	101	+1	92	92	0	352	434	+23.6
MS†	None	95.0	51.0	8.3	8.0	-0.3	77	77	0	116	129	+13	72	84	+16.7	347	356	+2.6
MS‡	None	98.5	51.0	7.6	8.0	+0.4	63	77	+14	125	129	+4	76	84	+10.5	316	356	+12.6
KN‡	None	93.3	54.3	8.6	9.3	+0.7	72	80	+8	102	101	-1	111	117	+5.4	402	483	+20.1
DV‡	Emphysema	105°C	57.5	8.3	8.4	+0.1	91	98	+7	94	90	-4	105	129	+22.8	371	450	+29.4
NG‡	Emphysema	108°C	46.1	9.4	9.9	+0.5	82	94	+12	89	86	-3	107	114	+6.5	276	356	+29.0

* Mean of consecutive five-minute observations.

† Mean of three observation periods, uncorrected to surface area.

‡ Control periods preceded anoxic periods.

§ Anoxic periods preceded control periods.

|| Per cent hemoglobin saturation while breathing 100 per cent oxygen. Per cent saturation used in this instance because the measurement of tension by Riley's method is inaccurate at tensions over 110 mm. Hg. The values appear greater than 100 per cent because of the oxygen dissolved in the plasma.

TABLE II
Effect of anoxia on renal excretion of electrolytes
All values represent the mean of three consecutive periods

Subject	Sodium			Chloride			Urine flow			Potassium			Phosphorus			Ammonia			pH		
	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change	Control	Anoxia	Change
CH*	346	397	+ 51	299	388	+ 89	2.35	2.01	- 0.34	72	81	+ 9	27.8	74.0	+46.2	11.5	4.3	- 7.2	7.6	7.3	- 0.3
LS*	216	278	+ 62	206	241	+ 35	5.18	4.17	- 1.01	64	58	- 6	4.6	10.0	+ 5.4	24.0	22.9	- 1.1	6.8	7.0	+ 0.2
LB*	92	186	+ 94	70	176	+ 106	0.50	0.84	+ 0.34	50	56	+ 6	18.9	23.6	+ 4.7	18.0	22.0	+ 4.0	5.2	5.3	+ 0.1
NH*	141	338	+ 197	202	286	+ 84	2.04	2.43	+ 0.39	109	76	- 33	15.4	21.2	+ 5.8	15.8	7.1	- 8.7	6.4	7.0	+ 0.6
MS*	152	243	+ 91	131	241	+ 110	0.81	1.24	+ 0.43	60	80	+ 20	24.4	35.6	+ 11.2	7.2	5.8	- 1.4	6.4	7.0	+ 0.6
MS†	132	243	+ 111	118	241	+ 123	0.60	1.24	+ 0.64	43	80	+ 37	40.2	35.6	- 4.6	6.6	5.8	- 0.8	5.3	7.0	+ 1.7
KN†	191	234	+ 43	182	217	+ 35	0.95	4.45	+ 3.50	47	67	+ 20	11.2	8.8	- 2.4	20.4	18.6	- 1.8	6.3	7.1	+ 0.8
DV†	90	163	+ 73	112	136	+ 24	0.67	1.17	+ 0.50	36	76	+ 40	6.3	3.9	- 2.4	31.5	12.0	- 9.5	6.0	7.6	+ 1.6
NG†	95	222	+ 127	132	282	+ 150	1.08	3.07	+ 1.99	38	91	+ 53	19.9	23.2	+ 3.3	33.7	27.4	- 6.3	4.6	5.0	+ 0.4
Mean	162	256	+ 94	161	245	+ 84	1.57	2.29	+ 0.72	58	74	+ 16	18.7	26.2	+ 7.5	17.6	14.0	- 3.6	6.1	6.7	+ 0.6

* Control periods preceded anoxic periods.

† Control periods followed anoxic periods.

chloride. Urinary pH shifted to higher values in all observations except one but this subject had a control value of pH 7.6. Ammonia production decreased in all but one observation. Potassium excretion increased in all but two observations. Proteinuria did not develop during anoxic periods.

The phosphorus excretion usually increased as the experiment progressed irrespective of changes in blood oxygen tension.

DISCUSSION

A lowered arterial blood oxygen tension in subjects with normal cardiovascular function induced a marked increase in the renal excretion of sodium and chloride. In no instance did renal plasma flow decrease during anoxia. It would appear, therefore, that anoxic anoxia per se is not the cause of the retention of salt and water nor in the reduction of renal plasma flow observed in congestive heart failure.

If arterial blood oxygen tension is lowered in normal subjects, renal plasma flow and sodium and chloride excretion increase. If arterial oxygen tension is raised in anoxic emphysematous subjects, renal plasma flow and the excretion of sodium and chloride decrease.

The duration of anoxia was only one to two hours in the normal individuals in the investigation, but the emphysematous individuals had maintained a status of anoxic anoxia over a period of years. It is of interest that even in the latter subjects the effect of chronic anoxic anoxia on the kidney was readily altered.

The effect of lowering arterial blood oxygen tension beyond 50 mm. Hg was not investigated as complicating changes in cardiovascular and pulmonary dynamics were likely to be introduced. It is to be noted that in congestive heart failure and in disease states associated with arterial oxygen unsaturation, oxygen tensions seldom fall below 50 mm. Hg.

In the normal subjects during anoxia, oxygen tension in inspired air was 106 mm. Hg. McDonald and Kelley (17) investigated renal plasma flow and filtration rate in dogs at simulated altitudes where oxygen tensions in inspired air were 79.4 and 61.6 mm. Hg. They found renal plasma flow to increase at higher altitudes, observations which are in accord with those reported here.

The increase in urine flow in these studies confirms previous work in the rat (18), dog (19), and man (20) where polyuria has been observed at higher altitudes. Burrill *et al.* (21) reported a rise in the excretion of sodium and chloride during one hour exposures of adult males to simulated altitudes of 18,000 feet. Interestingly enough, they observed a compensatory retention of these ions following the exposure so that the 24 hour excretion was not altered.

The normal dog excretes excessive amounts of sodium and chloride when exposed to 10.5 per cent oxygen for 24 hours whereas the adrenalectomized animal fails to do so (22). Thorn (23) found rats treated with adrenal cortical extract survived longer at higher altitudes than untreated animals. These facts suggest the possibility that the changes in sodium and chloride observed in the present study may be in some way related to altered adrenal metabolism at low oxygen tensions. The behavior of potassium did not, however, conform to this hypothesis.

SUMMARY

Renal plasma flow in man did not decrease under situations of anoxia where arterial oxygen tension was lowered to about 50 mm. Hg. The most pronounced effect of lowered oxygen tension on renal tissue was the increased excretion of sodium and chloride. Anoxic anoxia alone, therefore, does not appear to be the cause of the reduction of renal plasma flow nor the retention of salt and water observed in congestive heart failure.

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DETERMINATION OF ADRENERGIC AND CHOLINERGIC SUBSTANCES IN THE BLOOD OF MAN¹

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Much has been written about the clinical manifestations which indicate disturbances in function of the autonomic nervous system in numerous disease processes. Various clinical signs are identified with orthosympathetic or parasympathetic predominance. Autonomic drugs are used diagnostically and therapeutically, and surgical procedures have been devised which temporarily or permanently interrupt the neurogenic influences of this system upon the various parts of the body. Yet, consistently lacking have been objective tests which might indicate the nature or degree of existing autonomic disturbances. The availability of such tests would materially facilitate an evaluation of the importance of the autonomic nervous system in such diseases as thyrotoxicosis, peptic ulcer, hypertension, bronchial asthma, and a large number of the neuropsychiatric disorders.

Current concepts of the mediation of autonomic nervous system effects include humoral mechanisms. Investigation of the latter was the object of this study.

The well known responses of isolated rabbit intestine to epinephrine and acetylcholine have made possible its use as a method of identification of these substances in various solutions, including blood. The marked sensitivity and the relative specificity of the adrenergic response (1) as well as the simplicity of the method make it the best of the biologic tests for this substance so far developed. The cholinergic response is equally sensitive but much less specific since many substances besides acetylcholine cause an increase in the tone and contraction of the gut by their action upon the parasympathetic ganglion cells in the walls of the muscle (2, 3).

Isolated rabbit duodenum was used by Milhorat, Small, Doty and Bartels (4) and Diethelm, Doty and Milhorat (5) to assay the blood of patients with emotional disturbances for adrenergic

and cholinergic substances. They concluded that anxiety, resentment, and anger appeared to be accompanied by adrenergic factors in the blood while tension and possibly fear were accompanied by cholinergic factors. Furthermore their results indicated no essential difference between normal and psychopathological emotions except in the intensity of adrenergic and cholinergic responses.

If it is true that adrenergic and cholinergic substances can be demonstrated in the blood of healthy individuals, one might expect qualitative or quantitative differences of these substances in patients with diseases which are accompanied by evidence of disturbed autonomic function. In addition, autonomic nervous system surgery might be expected to alter these circulating substances, especially if the humoral mechanism of autonomic effect is significant. Two circumstances are recognized, however: 1. the human organism is capable of maintaining a remarkably fine humoral balance under normal and some abnormal conditions, and 2. humoral influences are effected not only by the amount of a circulating material but also by the ability of a tissue to respond to it.

PROCEDURE

Rabbits were killed by the injection of air into an ear vein. The intestine was cut at the pylorus and about 10 cm. of the duodenum removed. After thorough washing a piece 1.5 cm. in length was placed in 200 cc. of Ringer-Tyrode solution maintained at a temperature of 36° to 38° C by a thermostatically controlled water bath. The solution was stirred gently by a stream of air. A recording lever was attached to one end of the muscle and the contractions were recorded on a kymograph. The sensitivity of the preparation to epinephrine and acetylcholine was determined by the addition to the solution of these drugs in varying amounts by means of long screw-controlled pipettes. An amount was chosen which would give consistently a decisive but not prolonged effect. For acetylcholine this meant a concentration of 1:200,000,000 to 1:50,000,000; for epinephrine it varied from 1:100,000,000 to 1:16,000,000. The acetylcholine was added first, followed by the epinephrine, after which the preparation was washed and resuspended in fresh Ringer-

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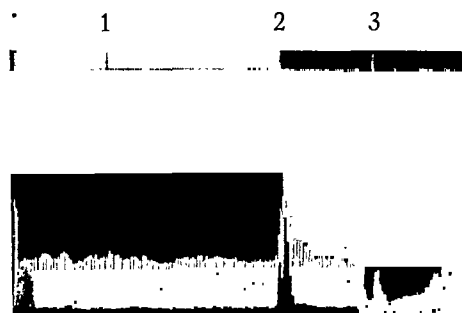


FIG. 1

1. Blood from normal male added—negative response.
2. 0.15 cc. ACH, 1:10,000 added.
3. 0.12 cc. EPH, 1:10,000 added.

Tyrode solution. If epinephrine was added to a preparation repeatedly without changing the Ringer-Tyrode solution, the sensitivity of the tissue decreased with each successive application until finally the contractions continued with the usual amplitude and frequency despite the addition of the drug. If, however, the tissue was then washed and resuspended in fresh solution, it again responded with its original sensitivity. This change in sensitivity with repeated applications of the drug has been noted by Hoskins (1) and Bernheim (6) although neither offered an explanation for the phenomenon.

When the response of the preparation to known solutions of epinephrine and acetylcholine was determined, blood specimens were tested as follows. The blood was drawn from the patient's median cubital vein into a syringe moistened with heparin and within three to 10 minutes was added to the fresh Ringer-Tyrode solution in which the muscle was suspended. After the effect of the blood was recorded, known amounts of epinephrine and acetylcholine were added to this solution to test the tissue sensitivity. Thereafter, the tissue was again washed and suspended in fresh Ringer-Tyrode preparatory for

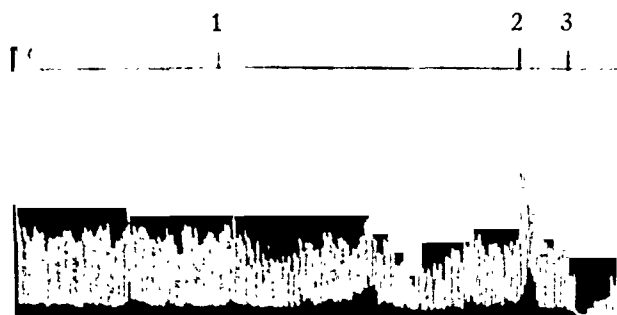


FIG. 2

1. Blood from normal male added—indeterminate response.
2. 0.02 cc. ACH, 1:10,000 added.
3. 0.02 cc. EPH, 1:10,000 added.

another test. It was possible to use most muscle preparations for four to six hours with no significant change in their response to the known amounts of epinephrine and acetylcholine. In some instances, in addition to the whole blood sample, blood was centrifuged, separated, and the plasma and cells tested separately. In these latter experiments, there was an interval of 35 to 45 minutes between the drawing of the blood and the addition of the separate constituents. Test solutions of varying concentrations of pitressin were also used.



FIG. 3

1. Blood from normal male added—CHO (2) response.
2. 0.04 cc. ACH, 1:10,000 added.
3. 0.04 cc. EPH, 1:10,000 added.

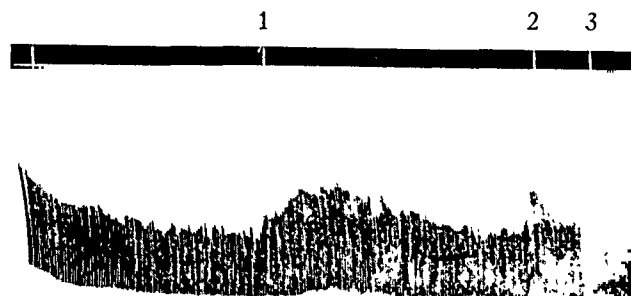


FIG. 4

1. Blood from hypertensive female added—prolonged CHO effect.
2. 0.04 cc. ACH, 1:10,000 added.
3. 0.04 cc. EPH, 1:10,000 added.

Responses were recorded as follows: "cholinergic" (CHO), meaning an increase in muscle tone and amplitude of contraction; "adrenergic" (ADR), meaning a decrease in tone and amplitude of contraction; "Negative" (NEG), if no change was noted; and "indeterminate" (I), when the change was bizarre and neither definitely cholinergic nor adrenergic. The degree of response was recorded as 1. (less than), 2. (equal to), and 3. (more than) the response of the tissue to known amounts of acetylcholine and epinephrine.

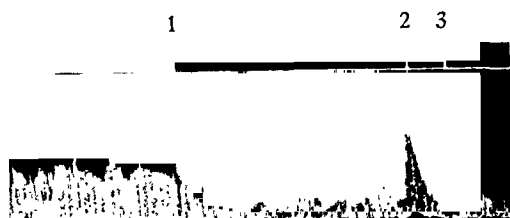


FIG. 5

1. Blood from male with ulcerative colitis added—prolonged ADR effect.
2. 0.04 cc. ACH, 1:10,000 added.
3. 0.08 cc. EPH, 1:10,000 added.

Responses which did not occur within 25 seconds after the addition of the blood were considered "delayed" (D), and those persisting more than five minutes were considered "prolonged" (P). In no instances did responses to known amounts of the pure drugs persist longer than one minute.

In some instances the response was at first cholinergic and then adrenergic. This is so indicated by an arrow in the tables of results. Examples of the various responses are shown in Figures 1 through 7.

The types of cases studied are listed in Table I. Controls consisted of members of the hospital staff and were engaged in essentially the same duties. All were well known and were felt to be emotionally stable. Each was questioned regarding the existence of any particular or excessive emotional reaction existing at the time the blood sample was taken. Patients were selected as typical and clear-cut examples of the illnesses from which they suffered. Those with emotional disturbances had chronic anxiety tension reactions which varied but little from day to day. In each instance, at the time of testing, none was experiencing any acute or unusual emotional reaction.

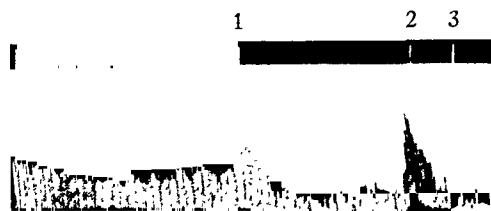


FIG. 6

1. Blood from female with asthma added—CHO effect followed by ADR effect.
2. 0.04 cc. ACH, 1:10,000 added.
3. 0.08 cc. EPH, 1:10,000 added.

TABLE I

Types of cases studied

20	Normals
3	Ulcerative colitis
3	Peptic ulcer
3	Asthma
8	Thyrotoxicosis
11	Anxiety-tension state
1	Schizophrenia
1	Involuntional melancholia
8	Essential hypertension
58	Total

Morning and afternoon blood specimens were drawn two to three hours after food ingestion and in the majority of cases were taken on the same day.

RESULTS

The response of the muscle strip to whole blood from normal subjects (Tables II and III) was varied and highly irregular, both in the group as a

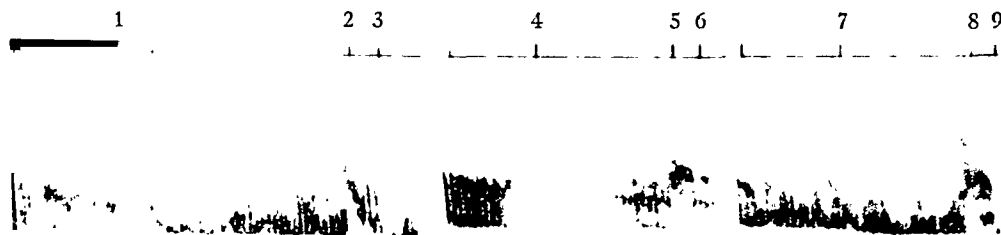


FIG. 7

1. Whole blood from male hypertensive (post-sympathectomy) added—response indeterminate.
- 2, 5, and 8. 0.04 cc. ACH, 1:10,000 added.
- 3, 6, and 9. 0.12 cc. EPH, 1:10,000 added.
4. Plasma of same blood added—ADR followed by CHO response.
7. Cells from same blood added—ADR response.

TABLE II

Response of muscle to whole blood of normals

Subjects	Morning	Afternoon
1 Female	ADR (3) P	CHO (2)→ADR (3) P
2 Male	CHO (2)→ADR (1) P	CHO (2)→ADR (3) P
3 Male	I	ADR (1) P
4 Male	I	CHO (2)
5 Male	—	ADR (1) P
6 Female	—	ADR (2) D&P
7 Male	CHO (1)→ADR (2)	NEG
8 Male	NEG	NEG
9 Male	ADR (2) D&P	NEG
10 Male	NEG	NEG
11 Male	NEG	ADR (2) P

whole and in the individual subject. This is especially apparent in the morning and afternoon samples tested on consecutive days. Types of response could not be correlated with any particular environmental situation, physical state, or minor emotional reaction arising during the course of the subject's daily hospital duties.

No consistent responses were observed when whole blood, plasma, or cells were tested separately (Table IV and Figure 7). Cannon (7) reported that serum and whole blood would cause the same shortening when added to the muscle strip. Mil-

horat *et al.* (4) stated that the substances in the blood producing the effects they described were labile and would disappear if the blood was allowed to stand 15 to 20 minutes after being drawn. Cannon (7) stated that blood from excited animals which produced inhibition of an intestinal strip lost this power upon standing in the cold for 24 hours or when kept warm and agitated with bubbling oxygen for two to three hours. As can be seen in Table IV, in only two instances did the addition of plasma fail to show an effect upon the muscle even though it was added as long as 30 to 45 minutes after withdrawal of the blood from the subject.

The blood from patients with chronic anxiety tension reactions (Table V) likewise showed considerable irregularity of effect. Morning and afternoon variation was marked; there was no relationship between the type and intensity of the muscle response and the severity of the emotional disorder; and in general, the intensity of the muscle response was less than the response to the solutions of known amounts of acetylcholine and epinephrine. As pointed out above, none of these patients was tested during acute emotional reactions. All exhibited chronic anxiety over their somatic symptoms and existing environmental problems, and were prone to develop excessive tension in response to stress. In the majority of them, psychiatric investigation had demonstrated the presence of deep-seated and repressed resentment and/or fear. With such mixtures of emotional reactions, individually accompanied by adrenergic or cholinergic factors (5), such variability of results as were observed was not unexpected. It is important to note that as a group they produced effects entirely similar to the controls.

One patient with a severe involutional melancholia was tested while she was extremely agitated, fearful, and protesting vehemently against the venipuncture. On one occasion (morning) her blood was adrenergic, on the other (afternoon) it was cholinergic. In each instance, it was impossible to determine the predominate emotional reaction.

Blood of patients suffering from peptic ulcer and ulcerative colitis, diseases in which parasympathetic predominance has been felt to exist, showed adrenergic effects in the majority of tests (Table V).

TABLE III

Response of muscle to whole blood of normals on successive days

Subjects	Day	Morning	Afternoon
12 Male	1	NEG	ADR (2) P
	2	—	ADR (1) P
13 Female	1	NEG	ADR (2) P
	2	NEG	ADR (1) P
14 Male	1	—	NEG
	2	—	NEG
	3	ADR (1) P	ADR (1) P
15 Female	1	ADR (1) P	—
	2	CHO (1)→ADR (1) P	ADR (2) P
16 Male	1	ADR (1) P	—
	2	NEG	ADR (1) P
17 Male	1	—	CHO (1)
	2	CHO (2)→ADR (2) P	CHO (1)→CHO (3) P
18 Male	1	—	NEG
	2	CHO (2)→ADR (1) P	NEG
	3	ADR (1) D&P	CHO (1)→ADR (1) P
	4	ADR (1) D&P	—
	5	—	CHO (1) D
	6	—	ADR (2) D
	7	CHO (1)	—
19 Male	1	—	NEG
	2	—	ADR (1) D
	3	I	CHO (2)→ADR (1) P
	4	NEG	ADR (1) P
	5	NEG	—
	6	I	NEG
20 Female	1	—	NEG
	2	ADR (1) P	ADR (1) P
	3	NEG	ADR (1) P
	4	NEG	ADR (1) P
	5	NEG	NEG

TABLE IV

Effect of whole blood, plasma and cells from normal subjects and patients on one or more days

Subjects	Diagnosis	Day		
18 Male		1	Blood Plasma Cells	ADR (1) CHO (1) ADR (1)
		2	Blood Plasma Cells	NEG CHO (1) D&P I
19 Normal female		1	Blood Plasma Cells	CHO (2)→ADR (1) P NEG ADR (1) P
20 Normal male		1	Blood Plasma Cells	ADR (1) P CHO (2) P CHO (2)→ADR (1) P
		2	Blood Plasma Cells	I CHO (2) P ADR (1)
		3	Blood Plasma Cells	ADR (2) P NEG ADR (1)
		4	Blood Plasma Cells	ADR (1) P ADR (1) P I
34 Female	Hypertension	1	Blood Plasma Cells	CHO (2) ADR (2) I
35 Female	Hypertension	1	Blood Plasma Cells	CHO (2) P ADR (2) ADR (1)→CHO (2) P
37 Female	Hypertension, preoperative postoperative postoperative		Blood Blood Plasma Cells	CHO (2) D&P I ADR (3) ADR (2) D
38 Male	Hypertension, preoperative postoperative postoperative		Blood Blood Plasma Cells	NEG CHO (1)→ADR (1) ADR (3) CHO (1)→ADR (1)

Blood from bronchial asthma patients was predominately adrenergic in effect, regardless of the severity of symptoms at the time of specimen withdrawal. The type of muscle response—delayed and prolonged—was more consistent in this group than in any other studied. No explanation for this was apparent. All blood samples were drawn at least two hours after any medication. One patient (No. 3), who in addition to asthma had a severe anxiety tension state, could not tolerate epinephrine.

Results from thyrotoxic patients were equally as irregular as the control studies. Degree of toxicity did not seem to be a factor.

Likewise there was considerable variability in the effects of blood taken from patients suffering from essential hypertension. In three cases (Nos. 36, 37, and 38) samples drawn the day before and seven days after thoracolumbar sympathectomy were similarly inconsistent.

In all of the experiments, the response of the muscle strip to blood was frequently prolonged and in this respect differed considerably from the response to solutions of acetylcholine and epinephrine.

DISCUSSION

The data obtained allow for no conclusions regarding the status of the autonomic nervous sys-

TABLE V
Response of muscle to whole blood of patients

Pt. no.	Sex	Diagnosis	Morning	Afternoon
1	M	Anxiety-tension state	NEG	CHO (1)
2	F	Anxiety-tension state	NEG	CHO (1)
3	F	Anxiety-tension state	NEG	ADR (1) D&P
4	F	Anxiety-tension state	—	ADR (1) D&P
5	M	Anxiety-tension state	ADR (1) D&P	—
6	M	Anxiety-tension state	—	ADR (1) D&P
7	M	Anxiety-tension state	NEG	I
8	M	Anxiety-tension state, diabetes	—	NEG
			NEG	CHO (1) P
			CHO (1) P	—
			—	I
9	F	Anxiety-tension state	I	ADR (1)
10	F	Anxiety-tension state	—	I
11	F	Anxiety-tension state, rheum. arthritis	—	ADR (1) P
12	F	Involutional melancholia	NEG	—
13	F	Schizophrenia, catatonic type	ADR (1) P	CHO (1) P
			—	ADR (1) D&P
14	M	Ulcerative colitis	ADR (1) P	ADR (1) P
15	M	Ulcerative colitis	—	ADR (1)
16	F	Ulcerative colitis	NEG	—
			I	—
17	M	Peptic ulcer	NEG	ADR (1)
18	F	Peptic ulcer	—	ADR (1) D&P
19	M	Peptic ulcer, healed	—	NEG
20	M	Bronchial asthma	—	ADR (1) D&P
21	M	Bronchial asthma, peptic ulcer	—	ADR (1) D&P
22	F	Bronchial asthma	—	ADR (1) D&P
			CHO (1)→ADR (1) P	ADR (2) D&P
			NEG	—
			I	ADR (1) D&P
23	F	Thyrotoxicosis	I	I
24	M	Thyrotoxicosis, congen. heart disease	ADR (1) D&P	—
25	F	Thyrotoxicosis	I	ADR (1) D
26	M	Thyrotoxicosis	CHO (1)	CHO (1)
27	M	Thyrotoxicosis	NEG	NEG
28	F	Thyrotoxicosis	CHO (1) P	—
29	F	Thyrotoxicosis	—	I
30	F	Thyrotoxicosis	—	I
			NEG	I
31	M	Hypertension	NEG	—
32	F	Hypertension, conversion hysteria	ADR (2) P	ADR (1) D
33	F	Hypertension, A-T state	CHO (1) P	—
34	F	Hypertension	CHO (2)	—
35	F	Hypertension	CHO (2)	—
36	F	Hypertension (pre-sympathectomy) (7 days post-sympathectomy)	—	NEG
			—	NEG
37	F	Hypertension (pre-sympathectomy) (7 days post-sympathectomy)	CHO (2) D&P	—
			I	—
38	M	Hypertension (pre-sympathectomy) (7 days post-sympathectomy)	NEG	—
			CHO (1)→ADR (1)	—

tem in the several diseases studied. The most that can be said is that with the method used there is no significant difference in the effects upon isolated rabbit duodenum of blood taken from normal persons and patients suffering from certain types of chronic emotional disturbances, ulcerative colitis, peptic ulcer, bronchial asthma, thyrotoxicosis, and essential hypertension. It remains entirely

possible that in these and other diseases, substances may be present in the blood which are the result of, or are quantitatively altered by, disturbances in autonomic nervous system function. Should such substances be present, it must be concluded that the method under investigation is not capable of consistently demonstrating them.

Aside from the irregularity and inconsistency

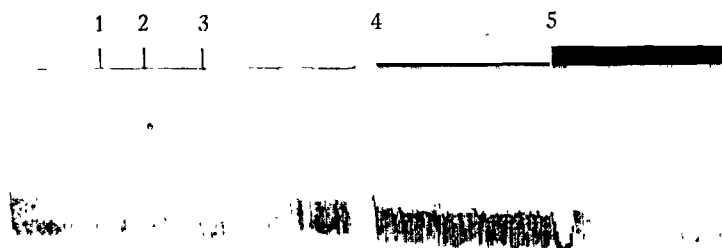


FIG. 8

1. 0.02 cc. pitressin diluted 1:1000—no effect.
2. 0.10 cc. pitressin diluted 1:1000—no effect.
3. 0.02 cc. pitressin undiluted (20 units/cc.)—ADR effect followed by CHO effect.
4. New Ringer-Tyrode solution.
5. 0.02 cc. pitressin undiluted—same effect as before.

of the results for which no satisfactory explanation is apparent, the very nature of the responses of the muscle to blood was sometimes difficult to interpret. In numerous tests, after the addition of the blood sample, the first effect noted was a transient increase in muscle tone and amplitude of contraction. This was followed by a decrease in tone and amplitude which might be marked and prolonged. This type of response might be interpreted as evidence of the existence of both cholinergic and adrenergic substances in the blood and in our results has been indicated as such.

However, Hoskins (8) observed that the application of epinephrine in a dilution subminimal to that needed to produce a characteristic inhibition of muscular activity would frequently increase the activity of the tissue and often cause an increase in tonus. Thus both types of muscle response might be produced by an adrenergic factor. In our experiments, especially in the initial mixing of blood with the Ringer-Tyrode solution in which the muscle was suspended, some transitory variation in dilution might possibly have occurred and could not have been avoided. It has also been shown that epinephrine appears to have the ability of increasing the synthesis of acetylcholine (9) and of inhibiting cholinesterase activity (10, 11). Presumably, under the proper circumstances, this also could result in an alteration of characteristic muscle response.

Reference has been made earlier to the ability of many substances besides acetylcholine to increase

the tone and amplitude of contraction of the gut. The response of the duodenal muscle strip to pitressin (Figure 8) is of interest in this regard. Actually, there is considerable doubt whether acetylcholine is ever present in circulating blood under normal circumstances. Existing in the tissues in a physiologically inactive form, it is activated by nerve impulses but almost immediately destroyed *in situ* by the enzyme, cholinesterase, which is a normal constituent in blood (12, 13). Dale (13) has shown that only in the presence of eserine does acetylcholine appear in a perfusion fluid. Thus it is most unlikely that the "cholinergic" effects noted in this and other similar experiments were due to acetylcholine.

The frequency with which blood caused effects quite different from the solutions of epinephrine and acetylcholine, the marked irregularity and inconsistency of the results, and the uncertainty of the nature of the substances producing the "adrenergic" and "cholinergic" effects raise considerable question as to the significance of the response of isolated rabbit duodenum to blood. It becomes apparent that this method is not a satisfactory means of testing for the presence of truly adrenergic substances in the blood.

CONCLUSIONS

1. No significant differences were observed in the responses of isolated rabbit intestine to blood from normal, psychoneurotic, and organically ill

subjects. Marked variability and irregularity of responses were present throughout.

2. Repeated studies on the same subjects showed diurnal as well as day to day variations in effect. These variations did not correlate with the existing emotional status, the influence of environmental factors, or the type or severity of illness.

3. The irregularity and inconsistency of the results and the uncertainty of the exact nature of the substances in blood having what have been thought to be adrenergic and cholinergic effects upon muscle, cause us to feel that the response of isolated rabbit duodenum to blood is not a satisfactory method of studying autonomic nervous system function.

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CONCENTRATION-TIME COURSE IN THE PLASMA OF MAN OF RADIOMERCURY INTRODUCED AS A MERCURIAL DIURETIC¹

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INTRODUCTION

During the course of investigations into the mechanism of congestive heart failure and other states of edema, it became evident that there was need for further understanding of the pharmacodynamics of the mercurial diuretics, including their distribution, mechanism of action, and elimination. Studies based upon chemical methods of analysis are limited because of the minute concentrations of the mercurial compounds present in the body fluids and tissues and the number of samples which can be analyzed in a given time. The use of the tracer technic made possible the extension of these studies to smaller quantities and to a greater number of determinations.

This presentation is directed at a better definition of the concentration-time course of mercury intravenously injected as a diuretic into the circulating plasma of man. A mercurial diuretic (Mercuryhydrin⁴) labeled with radioactive mercury⁵ was employed in the tracer studies. It is well to realize that it is the mercury which is being traced and not the molecule of the diuretic.

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⁴ The sodium salt of methoxyoximercuripropylsuccinylurea with theophylline, prepared with radiomercury in this laboratory by Messrs. Harold Krahnke, Darwin Kaestner and Edwin Sprengler through the courtesy of Dr. H. L. Daiell, Director of Research, of Lakeside Laboratories, Milwaukee.

⁵ Radioactive mercury ($\text{Hg}^{203,201}$) was obtained from the Isotopes Division of the Oak Ridge National Laboratories.

MATERIALS AND METHODS

Fifteen subjects were studied. All except one were patients on the medical wards at the Charity Hospital. Certain clinical information concerning these subjects is presented in Table I.

The subjects were permitted to rest comfortably in bed in an air-conditioned room for 30 to 60 minutes in order to attain a stable state. Whenever the subject's clinical condition permitted, samples of urine were collected by means of a bladder catheter.

Two cc. of the radioactive mercurial diuretic, containing 78 mg. of total mercury, was injected intravenously over a period of about 20 seconds. Collection of blood samples was begun simultaneously with the beginning of the injection. Two types of collections were made: 1. In some subjects blood was withdrawn, by means of a syringe and a heparinized 20-gauge needle, from an antecubital vein contralateral to that into which the injection had been made. For the first two to three minutes, samples were collected as rapidly as 2 cc. of blood could be withdrawn (approximately four to eight seconds). The interval between collections was progressively increased every five to 10 minutes until at the end of one hour, collections were being made every 15 to 30 minutes for the remainder of the study, which lasted 100 to 180 minutes in all. 2. In another group of subjects, blood was taken from a femoral artery. It was allowed to flow freely from the open needle for the first two to three minutes, the time between collection of 2 cc. samples varying from two to five seconds. This interval was then progressively lengthened as described for the venous collections.

The blood samples were centrifuged and 0.3 cc. of the serum was dropped upon a filter paper disc, allowed to dry and cemented to a metal disc for counting.

Urine was collected as rapidly as samples of sufficient quantity for counting could be obtained. There was usually an interval of five to 15 seconds between collections. After the period of rapid collections of the blood samples, a sample of urine was obtained as nearly coincident as possible with the time of each blood sample.

Radioactive measurements were made with thin mica-window Geiger-Müller counter tubes or a continuous gas-flow counter tube. All measurements were recorded as counts per minute per cubic centimeter of fluid and were corrected to values corresponding to an injected dose of radiomercury with an activity of 10,000,000 counts per minute.

TABLE I
Summary of clinical data of the 15 patients studied

Subject	Age	Sex	Weight	Diagnosis	Status	Severity of edema	Previous mercurial diuretic
1	58	M.	—	Cirrhosis with ascites	Stationary	2+	None
2	40	F.	153	Hypertensive cardiovascular disease, congestive heart failure	Stationary	3+	Four days prior
3	64	M.	160	Hypertensive cardiovascular disease, congestive heart failure	Improving	3+	None
4	59	F.	139	Hypertensive and syphilitic cardiovascular disease; congestive heart failure	Worse on admission; improved after mercurial diuretic	4+	None day before
5	53	M.	140	Syphilitic heart disease, congestive heart failure	Improving	0-1+	None
6	58	M.	154.5	Arteriosclerotic heart disease, congestive heart failure	Rapidly improving	3+	None
7	34	M.	120	Congenital heart disease; unilateral lymphedema, right leg	No failure; stationary	4+ right leg	None
8	21	F.	92	Cervical lymphadenitis	Improving	0	None
9	33	M.	155	Pulmonary abscess	Recovered	0	None
10	60	F.	121.5	Diabetes	Controlled	0	None
11	34	M.	176	Chronic malaria	Recovered	0	None
12	21	F.	106.5	Postpneumonia	Recovered	0	None
13	61	M.	135.5	Lymphoma	Stationary	0	None
14	32	M.	126.5	Peptic ulcer	Recovered	0	None
15	34	M.	156.5	Amebic colitis	Improving	0	None

DATA AND METHODS OF ANALYSIS

Since the experimentally observed values of this study could not be made to fit precisely any regularly shaped curve, it may be considered better to abandon the idea of ever being able to define the regression of mercury from the blood until the methods of biologic analysis have been considerably improved. To take this course would undoubtedly remove criticism from the analyses and would eliminate the development of erroneous concepts. However, there would be little purpose in the mere presentation of observed values of blood concentration for radiomercury. Furthermore, if the nature and significance of the mathematical analyses of the data observed are properly understood, and if it is also recognized that such analyses extremely oversimplify the physicochemical processes related to the regression in concentration of mercury, so that they represent only an attempt to approach the definition of these complex processes, no misconceptions should result and discussions should follow. The latter point of view has been accepted for the presentation of these data, and discussions will include mathematical expressions which are only *approximations* of the biologic processes involved in the observed data.

The mathematical approach to the analysis of blood regression curves of radiosodium in dogs and in guinea pigs has been presented in detail by Gellhorn and associates (1), and in man by workers in this laboratory (2). Again, this method has been employed as an arbitrary approach to the analysis of the concentration-time course of radiomercury in man. The observed values were plotted on semilogarithmic paper and a close-fitting curve (frequently referred to below as the "mother" curve) was drawn in by inspection (Figure 1). The general equation for such a curve is:

tion for such a curve is:

$$C_p = A_1e^{-b_1t} + A_2e^{-b_2t} + A_3e^{-b_3t} + \cdots A_ne^{-b_nt}, \quad (1)$$

where C_p is the concentration of radiomercury in the serum at time (t).

$A_1, A_2, A_3, \cdots A_n$ = the respective amounts of the tracer which are regressing at the different rates.

$b_1, b_2, b_3, \cdots b_n$ = the rates of movement per minute of the respective amounts of the tracer out of the serum. The absolute values of these symbols indicate the portions of $A_1, A_2, \cdots A_n$, respectively, which leave the serum per unit of time.

There are numerous rates of movement or regression contributing to the observed curves, but because of the nature of the data and the curves, it was considered impractical to carry the analysis into more than three rates. During the interval of these studies the concentration of radiomercury in the serum continued to decline but never reached a constant value; therefore, the absolute value of b_3 in the third member of the equation was neither zero nor small enough to be considered as zero. This differs from the regression of sodium in that the concentration of the latter approached a constant value more closely. In fact, in the sodium studies such a value was considered for convenience to exist and, therefore, the C_{eq} value was employed, or b_3 was considered to equal zero (1).

The portion of the equation represented by the expression $A_2e^{-b_2t}$ was the tangent drawn through those points which most closely approached a horizontal and straight line in the "mother" curve (Figure 1). Selected values along this line extending to the ordinate ($t=0$) were subtracted from simultaneously occurring values along the "mother" curve. These differences were plotted, and a curve was drawn through the points. The expression of

the tangent to this new curve through the points which most closely approached a straight line was represented by $A_2e^{-b_2t}$. Selected values along this line extending to the ordinate were subtracted from simultaneously occurring values of the remainder of the curve not tangent to the line represented by $A_2e^{-b_2t}$; the differences were again plotted and a line was drawn through these points. The expression of this final "daughter" line, or regression rate, is represented by $A_1e^{-b_1t}$. It is evident from Figure 1 that it would have been impractical to carry the analysis further. Therefore, there appear to be at least three major rates of regression, each probably representing many physicochemical processes mathematically integrated to define the declination in concentration of mercury in the plasma of man. The sum of the values along these "daughter" curves at any time (t) represents the total concentration of the isotope in the plasma at that time, expressed graphically by Figure 1 or by equation (1). A discussion of the significance of these various regression rates will follow.

When the observed values from about two to about 200 minutes are plotted on logarithmic coordinates, they tend to lie along a straight line, the equation for which is $C_p = At^{-b}$, where

C_p = concentration of radioactive mercury in plasma at any time (t)

A = a constant which is numerically equal to the concentration of radioactive mercury in the plasma at one minute

t = time in minutes after injection

b = ratio of percentage change in concentration to percentage change in time (the slope of the curve on logarithmic coordinates).

Figure 2 permits the comparison of the curves of observed values for a subject plotted on rectilinear, semilogarithmic and logarithmic coordinates.

The regression curves of five of the 15 subjects studied were chosen for critical analysis. In these five the specific radioactivity of the plasma was sufficiently great to insure a high degree of accuracy in counting throughout the period of study. In Figure 3 these values are plotted on rectilinear (lower curves) and logarithmic coordinate (upper curves) systems. The regression curves or rates of regression of mercury from the plasma were similar for all 15 patients. Figure 4 illustrates a composite curve of all patients plotted on the semilogarithmic coordinate system. The degree of the

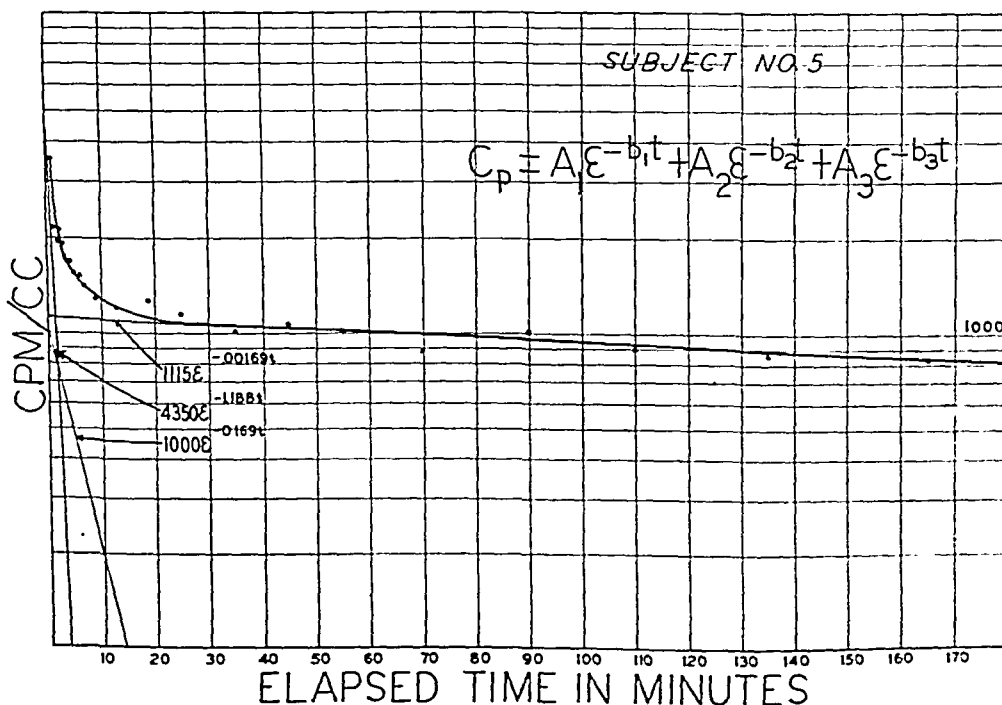


FIG. 1. THE OBSERVED POINTS AND MEAN CURVE OF THE CONCENTRATION-TIME COURSE OF RADIOMERCURY IN THE PLASMA OF SUBJECT 5

Three regression rates are shown and defined by the equation $C_p = 4350e^{-1.188t} + 1000e^{-0.169t} + 1115e^{-0.00169t}$.

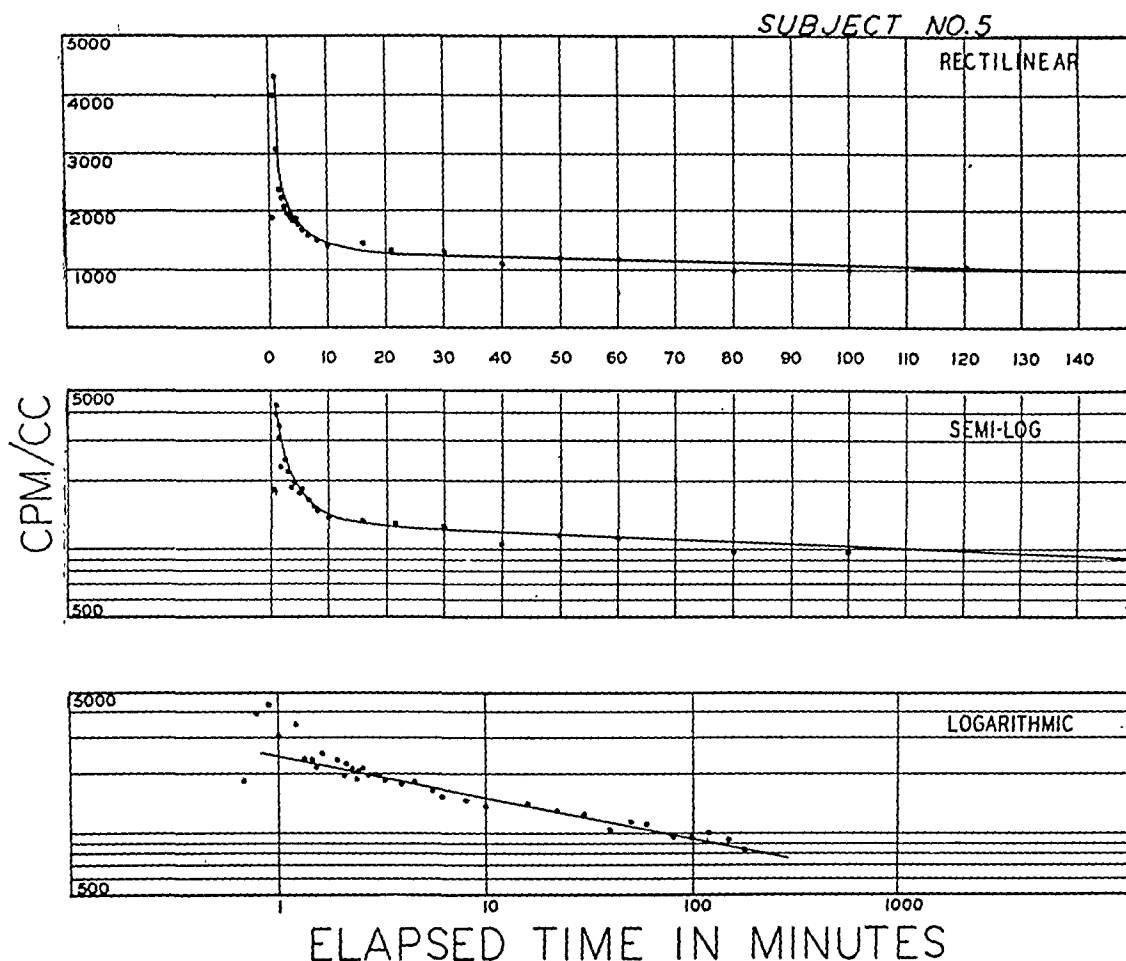


FIG. 2. CONCENTRATION-TIME COURSE OF RADIOMERCURY IN THE PLASMA OF SUBJECT 5
PLOTTED ON THREE DIFFERENT COORDINATE SYSTEMS

Note that a considerable number of the observed points tend to lie along a straight line drawn on the logarithmic coordinate system.

individual variations precluded a differentiation of the curves or rates of regression of one clinical state from that of another, since small differences would have no statistical significance in such a limited series.

Figure 5 illustrates the regression curve obtained in similar experiments with the use of Na^{22} as a tracer.⁶ The regression curves of one of the studies with radioactive mercury and one with radiosodium plotted on logarithmic coordinate system permit comparison of the concentration-time course of these elements in the plasma of man. The differences are self-evident, especially those existing after approximately 200 minutes.

⁶ These studies were conducted primarily for other purposes at Grady Hospital, Emory University Medical School, Atlanta, in collaboration with Drs. A. J. Merrill and Heywood Turner. The samples were collected in Atlanta and brought to New Orleans for counting.

DISCUSSION

The interpretation of the regression curves obtained for any tracer is complicated by the combination effects of simultaneously occurring phenomena. This is particularly true in the initial portion of the curves, since all processes concerned, rapid and slow, are manifested at this time, when the greatest changes in concentration are also taking place. Progressively the tracer recording those factors responsible for rates of rapid change approaches equilibrium level and manifests itself less and less in the completed regression curve.

As has been demonstrated previously for sodium (2), and in these studies for mercury, there are essentially three separate major regression rates which contribute to the decline in concentration of the tracer in the serum. Obviously, the *initial rate* of regression of the concentration of radio-

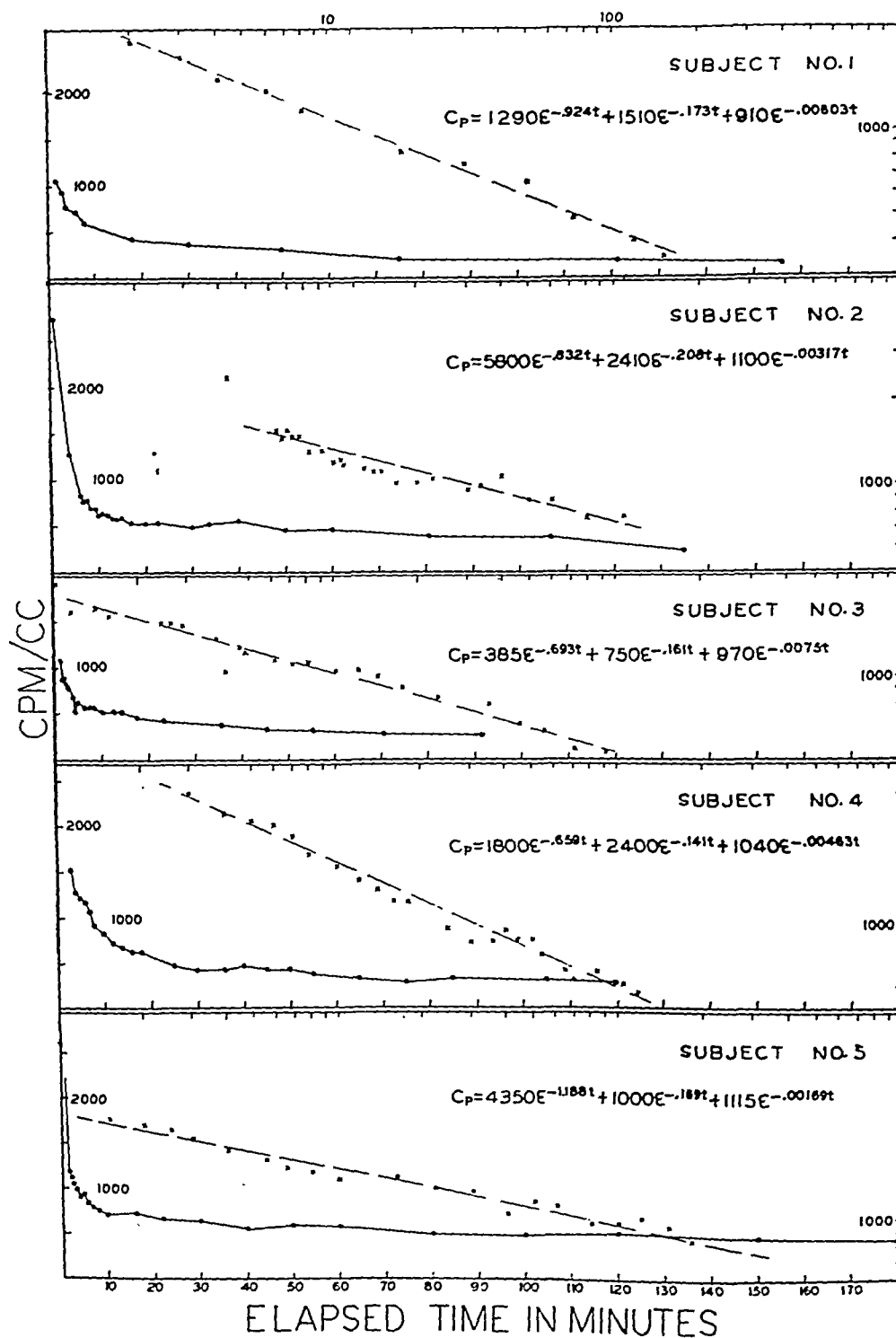


FIG. 3. THE CONCENTRATION-TIME COURSE OF RADIOMERCURY IN THE PLASMA OF SUBJECTS 1-5 PLOTTED ON BOTH RECTILINEAR AND LOGARITHMIC COORDINATES

The coordinates for the logarithmic graph are on the top and the right of each graph and for the rectilinear on the left and bottom. The equations shown were obtained by the semilogarithmic graphic method of analysis described in the text.

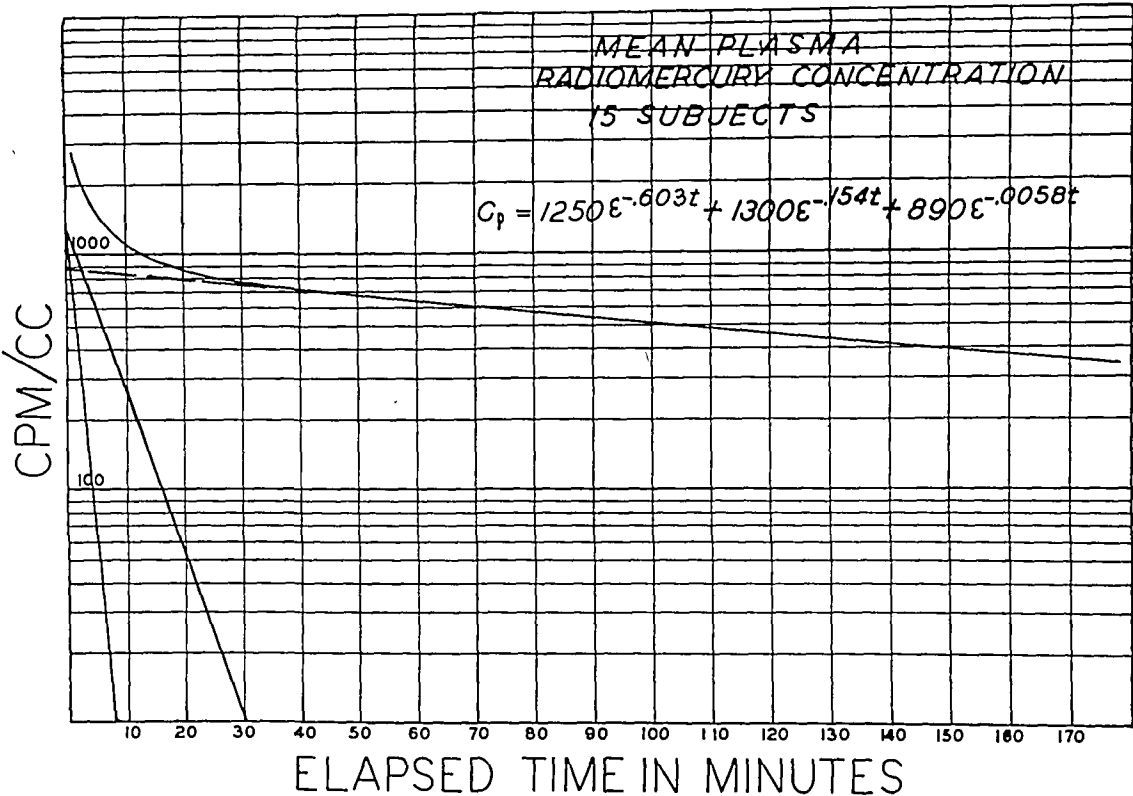


FIG. 4. THE MEAN OBSERVED CURVE AND EQUATION OF CONCENTRATION-TIME COURSE OF RADIOMERCURY IN THE PLASMA OF 15 SUBJECTS

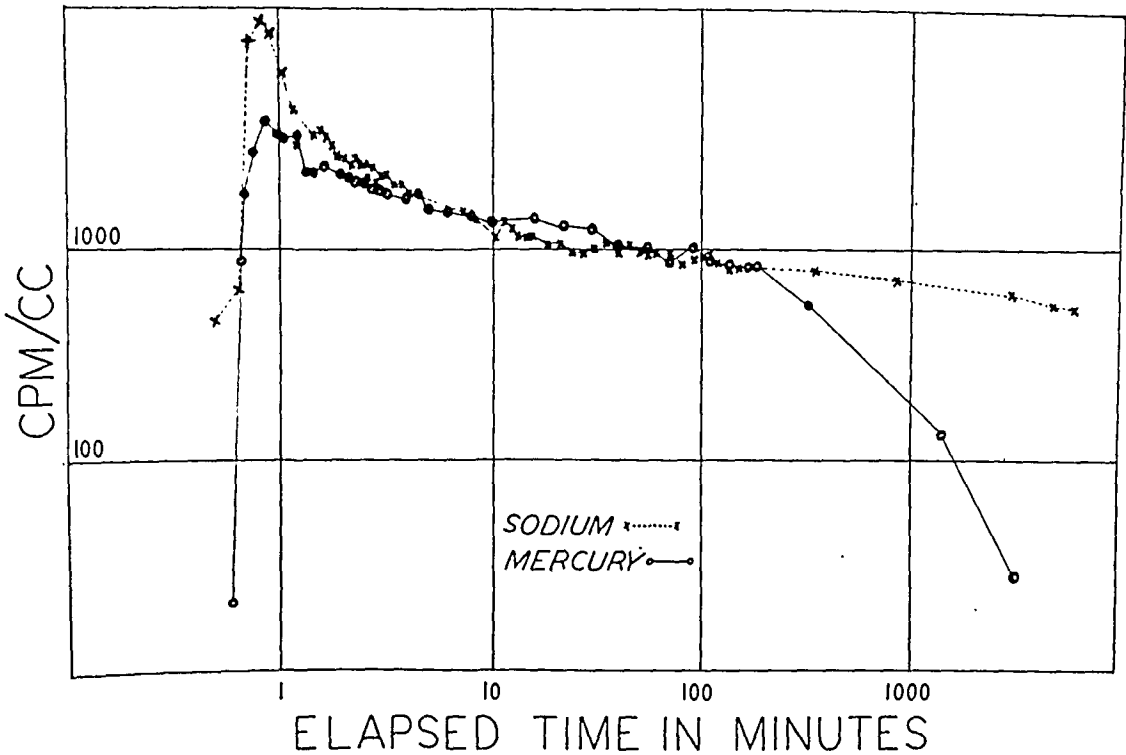


FIG. 5. THE PLASMA CONCENTRATION-TIME COURSE OF RADIOSODIUM COMPARED WITH THAT OF RADIOMERCURY IN TWO DIFFERENT SUBJECTS PLOTTED ON LOGARITHMIC COORDINATE SYSTEM

Note that whereas the curves are similar, they differ in the early portion, and that after 200 minutes the curves separate, the concentration of the mercury declining rapidly, with that of sodium being maintained. The ordinate values for both mercury and sodium were rendered comparable.

mercury in the plasma is influenced by the phenomenon of mechanical mixing. Figure 6 illustrates the ascension of concentration as well as the early portion of regression. A column of blood passing through the antecubital vein during the period of injection will contain a high concentration of radio-mercury. The local variations in concentration within the column will vary with the rate (force or linear rate and volume) of delivery of the radioactive substance. Since the collection of the blood samples begins simultaneously with the injection of the labeled substance, the first few samples collected will contain no radiomercury, the time of appearance of the tracer being dependent upon the circulation time and the rate and force of injection. When the tracer-containing blood column passes through the right side of the heart, its specific radioactivity is reduced by dilution with blood returning from other parts of the body; in the pulmonary vessels vigorous mixing takes place; and upon entering the left chambers of the heart, the radioactive column undergoes further mixing. Several ventricular contractions, at least as many as occurred during the time required for injection of the radio-mercury into the antecubital vein, are required to force the greater portion of the radioactive column of blood into the systemic circulation. From the moment the left ventricle receives radioactive blood, all blood leaving it will be radioactive because:

1. The column of blood into which the injection was made will contaminate the walls of all vessels through which it flows, which in turn contaminate in-flowing nonradioactive blood.

2. The blood which contains radioactive mercury ejected from the left ventricle will flow through vascular circuits of variable length, returning from the shortest (coronary circulation) before the return from the longest (lower extremity) has begun. This effects rapid, complete mechanical mixing and early, continuous return of radioactive blood to the left ventricle.

3. The linear velocity of blood flow through a vessel decreases from the center of the lumen of the vessel to the wall, the flow being almost zero at the surface of the wall. This phenomenon continuously increases the length of the column of radioactive blood, so that a continued flow of radioactive blood into the heart is maintained and mechanical mixing is encouraged. The process of

axial flow and surface skimming in vessels other than the segment receiving the injection will tend to delay mechanical mixing.

Under ideal experimental conditions the tracer substance should be completely mixed and distributed homogeneously in the blood at the instant of injection. A concentration-time curve would then be concerned with biologic phenomena other than mixing. Obviously, this ideal experimental condition cannot be achieved. An alternative approach might consist in delaying observations until mixing was completed; this, however, would be unsatisfactory because the movement of mercury into some compartments might be so rapid that the mercury in the blood might reach equilibrium with that in these compartments during the period allowed for mixing and before the observations are begun. The physiologic data available today strongly suggest that mixing has very little or no influence upon the regression curve after 30 minutes, probably negligible influence after 15 minutes in all subjects and even after one or two minutes in some subjects.

It becomes evident, therefore, that with rapid loss of a tracer substance from the blood and with relatively slow mechanical mixing, it is impossible to obtain an absolute picture of the true rate of loss of a tracer from the blood stream of man. It is also apparent that a true picture cannot be obtained from the later periods alone of the regression curve, because the tracer method is dependent upon the principle that all turnover phenomena, regardless of number and rate, are occurring at all times of study, special physiologic situations excluded.

It is safe to state that the initial portion of the concentration-time curve is the resultant of all the regression forces acting on mercury in the plasma stream of man and that the initial regression rate expressed as b_1 (Figures 1 and 4) is a manifestation largely of mixing processes occurring simultaneously with other rapid biologic phenomena of a physicochemical nature, such as fixation to protein molecules and cell surfaces. The other rates of regression are probably so little influenced by mixing that this phenomenon may be eliminated from serious consideration except insofar as mixing or continued circulation of the blood and other body fluids is necessary to insure satisfactory continuation of the other physicochemical processes

more directly responsible for the concentration-time course of mercury in the plasma.

The second regression rate is dependent upon those factors which are more apparent after the mixing process has been completed, for example, slower rates of diffusion into tissue spaces, cells, bone, and other sites of the body. Some of these have been discussed for sodium (3) and will not be repeated in detail.

It is well to realize that when Na^{24} or Na^{22} is employed as a tracer and is added to an organism such as man, it labels an already-existing, dynamic state of turnover of sodium among the various compartments within the body, *i.e.*, in the normal subject, except under special circumstances, the sodium space is already saturated with relatively large quantities of nontracer sodium. Therefore, the various components of the regression curves of tracer sodium represent its movement into sodium compartments already in a steady state concerned with active processes in dynamic equilibrium. Mercury, on the other hand, does not exist within the body under comparable conditions. That which is present normally in the body is apparently a contaminant and exists in relatively small quantities (4). It is possible, however, that

the "saturated" state for mercury in the body is zero concentration and that the body mechanisms are set to maintain this level. Nevertheless, there exists essentially a "turnover pattern" for mercury, which, unlike that for sodium, is not active. These innumerable turnover rates or regression processes for mercury manifest themselves only when the element is present, that which is normally present as a contaminant being disregarded. Thus, the radioactive mercury injected as an extremely small part of a total of 78 mg. of mercury in the mercurial diuretic labels practically all of the mercury which exists in the body (4) and which activates the "turnover patterns" for mercury. The regression curves of the radioactive mercury then must represent partially the migration of mercury into "depots" or "relatively fixed" compartments to fill "potential mercurial spaces." The result is an essentially unidirectional loss of mercury from the circulating serum rather than an exchange process already in equilibrium. The compartments into which mercury moves most probably include those systems concerned with the protection of the body from toxic effects of this foreign substance and with its subsequent excretion, for example, the liver, kidneys and cells and

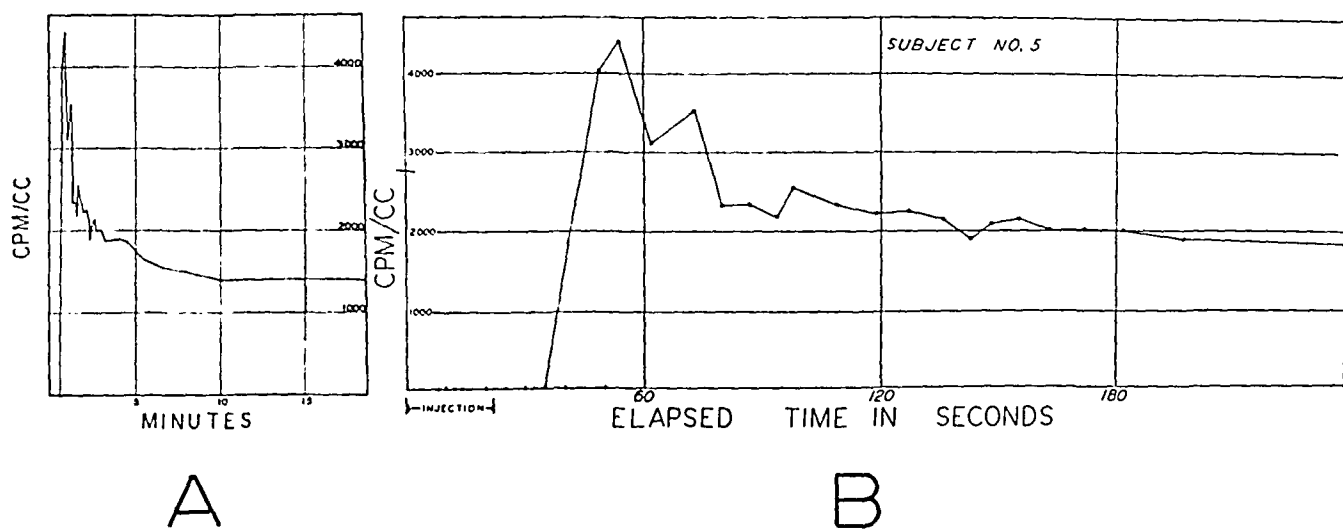


FIG. 6

A. The ascension and regression of the concentration of radiomercury in the plasma for the first 20 minutes after injection.

B. The first four minutes of curve A more clearly illustrate the early variations in concentration. Note that radioactivity first appeared in the blood of the femoral artery between 35 and 40 seconds after the beginning of the injection into an antecubital vein. Twenty-two seconds were required for the injection.

The intersection (extrapolated) on the ordinate at 2,800 c.p.m. indicates the concentration if mixing were complete at t_0 . Except for errors due to the loss of mercury from the plasma during the first three minutes, the ratio of the injected dose to this extrapolated concentration "indicates" the mercurial space which at t_0 is also the plasma volume, since mercury does not adhere to erythrocytes in significant amounts.

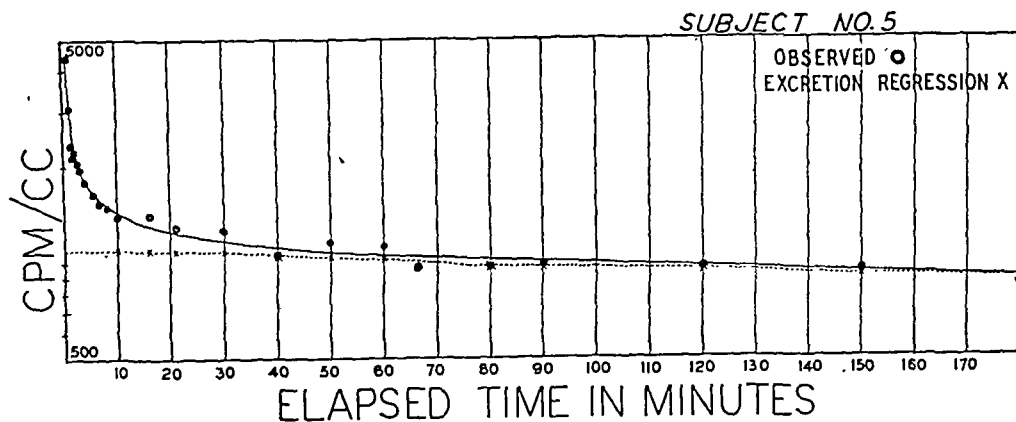


FIG. 7

The interrupted line indicates theoretic concentration of radiomercury in the plasma calculated from that excreted in the urine and compared with the observed concentration of radiomercury in the plasma.

protein compartments not in the blood stream. It is likely that the recorded regression phenomena for mercury would be entirely different if the mercury depots were already saturated and the turnover processes were progressing at full pace instead of existing merely as "potential turnover patterns."

After the relatively rapid phenomena have manifested themselves in the first two regression rates, the relatively slow excretory rate is more clearly observed as a significant part of the third regression rate (Figures 1 and 7). That this third rate of decline in concentration of the radioactive mercury in the serum is a reflection primarily of urinary excretion is supported by the fact that the urinary excretion appears to account for a considerable part of the loss of radiomercury from the serum of some subjects beyond approximately 60 minutes after the time of injection. In other subjects the urinary excretion may not account for as much of the decline but is responsible for a substantial fraction of the regression from the blood. Details of urinary excretion will be presented elsewhere. Other routes of excretion undoubtedly influence this portion of the curve also.

Since the segment of the curve of the concentration-time course of radiomercury in the interval from about two to 200 minutes appears to be almost linear on logarithmic coordinates (Figures 2 and 3), the ratio of the percentage change in concentration to percentage change in time is nearly constant in this interval. The significance of this has not been fully developed.

It should be pointed out that the differences in the regression curves of sodium and mercury (Figure 5) are probably due to the differences in the respective phenomena which they represent. The rate of regression of sodium is more rapid early and considerably less rapid later. This is expected, since sodium diffuses more readily but is excreted more slowly than mercury.

The relationships of concentrations of radioactive mercury in blood serum to those in other biologic fluids, including urine, are under study at the present time and will be discussed elsewhere. With better understanding of the nature of the regression of mercury from the plasma, other biologic studies employing mercury in man can be better planned and evaluated. Without detailed knowledge of the concentration-time course of mercury in the blood plasma, accurate experiments concerned with blood values would be impossible.

SUMMARY

The concentration-time course of a mercurial diuretic labeled with radioactive mercury and injected intravenously has been studied in 15 human subjects. Analysis of the regression curves demonstrates that there are at least three major exponential rates responsible for the decline in concentration of mercury in the plasma. The first rate is apparently dependent to a large extent upon mechanical mixing; the second is possibly due to relatively rapid adsorptive, chemical and diffusion phenomena with migration into dynamic and more stable compartments; the third rate is

most probably a reflection mainly of excretory processes, principally urinary. It was not possible from these data to draw any conclusions as to differences because of the various clinical states in the subjects studied. Comparison of these curves with those similarly obtained for sodium indicate that there may be slight differences in the early portions of the regression curves and that great differences exist in those portions of the curves dependent upon the excretion of the isotope. The concentration of sodium in the plasma practically reaches an equilibrium, whereas the concentration of mercury may reach a steady state but does not approach an equilibrium due, in part, to rapid elimination.

These observations make possible a better understanding of the pharmacodynamics of mercurial diuretics and certain physiologic phenomena and

thus facilitate better planning of experimental studies concerned with mercurial diuretics *necessarily based upon plasma concentration of mercury*.

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A STUDY OF CERTAIN ASPECTS OF BLOOD COAGULATION IN THE POSTOPERATIVE STATE IN CONGESTIVE HEART FAILURE AND IN THROMBOPHLEBITIS

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In recent years a number of tests have been designed for the purpose of detection of accelerated blood coagulation. Among these are the lusteroid tube coagulation test (1-4), the silicone tube coagulation test (5), the modified clot retraction test (6), the modified Lee-White clotting test, employing three test tubes instead of one (7), the heparin retarded coagulation test of Waugh and Ruddick (8, 9), the heparin tolerance test (10, 11), and the prothrombin time determination, using both whole and dilute plasma (12-14).

The study reported herewith was undertaken for the following purposes. First, it was desired to ascertain whether or not there is any correlation among the various tests themselves. Second, it was decided to determine whether any of these tests can be used to predict thrombophlebitis in patients. Finally, the incidence and duration of hypercoagulability in the postoperative state was studied, as well as the incidence of hypercoagulability in congestive heart failure. Incident to doing these studies, observations were made on the effect of variation of tube size and temperature upon the clotting time of blood in the Lee-White test. Further, in doing the heparin tolerance test, blood was withdrawn almost simultaneously from the vein into which the heparin was injected and from a vein of the opposite arm. Clotting times were run on each sample and the results were compared.

MATERIAL

For the study on postoperative patients, a group of 29 patients in the Cincinnati General Hospital who had had recent major surgical operations was selected at random. These 29 patients and three additional patients having acute thrombophlebitis were used to determine the correlation among the various tests. The problem of hypercoagulability in congestive heart failure was studied in 27

patients suffering from cardiac insufficiency on the medical wards of the Cincinnati General Hospital. A group of house officers, nurses, technicians, and ambulatory psychiatric patients were used as normal controls. Persons from both the control group and from the postoperative group were used to study the effect of variation in temperature and tube size upon the Lee-White clotting test. Persons from both groups were also used to study the effect of the veins chosen in performing the heparin tolerance test.

METHODS

The 29 postoperative patients and the three patients having acute thrombophlebitis were each studied by means of six tests which were run simultaneously. The same series of six tests was repeated on seven of the postoperative group later in their postoperative courses. The six tests employed were the modified Lee-White clotting test, the lusteroid tube coagulation test, the prothrombin time on whole and on 12.5% plasma, the Waugh-Ruddick heparin retarded coagulation test, and the heparin tolerance test. The 27 patients suffering from congestive heart failure were studied by means of the lusteroid tube coagulation test and the modified Lee-White test using three dry test tubes and an oiled syringe. Fifty of the control group were studied with the lusteroid tube coagulation test and the modified Lee-White test, using three test tubes and an oiled syringe. Twenty of the control group, all ambulatory psychiatric patients, were studied with the heparin tolerance test and the modified Lee-White test, using three saline-rinsed test tubes and a saline-rinsed syringe. The whole and dilute plasma prothrombin times were studied in 28 ambulatory psychiatric patients. The Waugh-Ruddick test was studied by means of 25 tests on a series of 15 persons in the control group.

PROCEDURE

1. Modified Lee-White test

This test was run essentially as described by Lee and White (15), with the exception that three test tubes were used instead of one. The second tube was not tilted until clotting had occurred in the first, and the third tube was not tilted until clotting had occurred in the second. The time required for clotting in the third tube was taken as the clotting time. This test was run in a constant tem-

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perature water bath at 37° C as suggested by Quick (16). This test was also run on each patient using three dry test tubes and an oiled syringe.

2. Prothrombin time on whole and 12.5% plasma

The method described by Quick (17) was employed, using Squibb thromboplastin.

3. Lusteroid tube coagulation test

The method used was similar to that of Kadish (1, 2), except that three tubes were used instead of one, the reading being taken from the third tube. The tests were run in a constant temperature water bath at 37° C. Blood was used only if obtained at the first attempt at venipuncture. Specimens were discarded if bubbling or frothing occurred in the syringe. The lusteroid tubes were 12.5 mm. in diameter. They were cleaned with sodium lauryl sulfonate, as recommended by Kadish (1, 2).

4. Waugh-Ruddick heparin retarded coagulation test

This test often shows acceleration of coagulation when the Lee-White test is normal. The method employed was that described by the original authors (8, 9), except that all tests were run in a water bath at 22.5° C, as recommended by Whittaker (18). This author found that a rise in temperature caused much more rapid clotting in the Waugh-Ruddick test; we have had a similar experience. Whittaker selected 22.5° as the average winter room temperature. Waugh and Ruddick did their tests at room temperature (8, 9). He found clotting times in normals comparable to those of Waugh and Ruddick when the tubes containing the blood were placed in a water bath at a temperature of 22.5° C (18). The criterion of increased coagulability in this test was that suggested by Ogura (19), namely, a coagulation time of 60 minutes or less in each of the last three test tubes employed.

5. Heparin tolerance test

Hagedorn and Barker's (11) modification of the test of deTakats (10) was employed. However, in consideration of the marked effect of temperature variation upon the clotting time of blood (16, 18), the tests were done in a constant temperature water bath at 37° C. Three test tubes, each 8 mm. by 75 mm., were employed.

As suggested by Hagedorn and Barker (11), one sample of blood was drawn 10 minutes after the injection of 25 mg. of heparin in 2.5 cc. of solution intravenously. The heparin was injected at a constant rate, requiring 30 seconds for its administration.

RESULTS

The findings in the control group will be given first. These are shown in Table I. In 50 controls, the range for the modified Lee-White test, using an oiled syringe, was from 9 to 23.5 minutes; the range for clotting in lusteroid was from 19 to 40 minutes in this group. In 20 controls, the range for the modified Lee-White test, using saline-rinsed glassware, was from 9 to 21 minutes. The mean for the 20 determinations was 11.58 minutes when saline-rinsed tubes and syringes were employed; the mean for 50 similar tests using an oiled syringe and dry test tubes was 14.77 minutes. The standard deviation for the 50 determinations using oiled syringes was 3.34 minutes. The standard deviation for the 20 determinations using a saline-rinsed syringe was 4.36 minutes. The standard error of the difference between the two means of 14.77 minutes and 11.58 minutes was found to be 1.08 minutes. The observed difference was 3.19 minutes. Since the observed difference in the means was three times the standard error of the difference, this was felt to be a significant finding. This difference would occur by chance only once in 370 tests. Ten minutes after the injection of 25 mg. of heparin intravenously, the modified Lee-White clotting time was increased from two to slightly more than four times that before heparin.

Among 28 control ambulatory psychiatric patients, the range for the prothrombin time run on whole plasma was from 11.5 to 16.00 seconds. The range for the prothrombin time on 12.5% plasma was 26 to 37 seconds; the range for the difference

TABLE I
Results of various coagulation tests in the control group

Name of test	Number of persons	Range	Mean
Lee-White (oiled syringe)	50	9 to 23.5 min.	14.77 min.
Lee-White (saline-rinsed syringe)	20	9 to 21 min.	11.58 min.
Heparin tolerance test	20	Clotting time increased 2 to 4 times	
Lusteroid tube clotting test	50	19 to 40 min.	
Prothrombin time (whole plasma)	28	11.5 to 16 sec.	13.5 sec.
Prothrombin time (12.5% plasma)	28	26 to 37 sec.	

TABLE II
Summary of findings in 29 postoperative cases

Operation	P. O. day	Pro. time whole	Pro. time 12.5%	Lee-White saline 37° C	Lust. 37° C	Heparin tol. 37° C	Waugh-Ruddick 22.5° C
1. Append.	6	0	0	0	0	0	acc
2. Hysterect.	6	0	0	0	0	0	acc
3. Nephrect.	6	0	0	0	acc	0	acc
4. Hernia	5	0	0	acc	0	0	0
5. Hernia	4	0	0	0	acc	0	acc
6. Nephrect.	1	0	0	0	0	acc	acc
7. Hysterect.	3	0	acc	acc	0	—	acc
8. Append.	4	0	0	0	acc	acc	acc
9. Hernia	4	acc	0	0	0	0	acc
10. Hernia	5	acc	0	0	acc	0	acc
11. Gunshot colon	3	acc	acc	0	0	0	acc
12. Gunshot colon	4	0	0	0	0	0	acc
13. Hernia	2	0	0	0	0	acc	0
14. Stab of abdomen	3	0	0	0	0	0	acc
15. Append.	2	0	0	0	0	0	0
16. Hysterect.	9	0	0	0	acc	—	acc
17. Hysterect.	3	0	0	0	0	0	acc
18. Append.	4	0	0	0	0	0	acc
19. Hernia	3	0	acc	acc	acc	0	acc
20. Hernia	4	0	0	0	0	0	0
21. Gunshot bowel	6	0	0	0	0	0	0
22. Gunshot bowel	2	0	0	0	0	0	acc
23. Stab of bowel	6	0	0	0	acc	acc	0
24. Stab of abdomen	2	0	0	0	acc	0	0
25. Append.	1	0	0	0	0	acc	0
26. Append.	1	0	0	0	acc	0	acc
27. Gunshot bowel	4	acc	0	0	acc	0	0
28. Hernia	2	0	0	0	0	acc	0
29. Perf. ulcer	6	acc	0	0	0	0	acc

Note: acc indicates acceleration
0 indicates no acceleration
— indicates that the test was not run

between the whole and dilute plasma prothrombin times was from 14 to 22 seconds.

Acceleration was considered present in any test when the result obtained was lower (*i.e.*, less time) than was found in any control test of the same method. Of the 29 postoperative patients, acceleration was found as follows: by Waugh-Ruddick, 19 showed acceleration; by lusteroid, 10; by heparin tolerance, six; by whole plasma prothrombin time, five; by 12.5% plasma prothrombin time, three; by modified Lee-White test run in saline-rinsed glass, three. The results are summarized in Table II. As seen in Table II, the correlation among the various tests was poor. Of 10 patients showing abnormal tests in lusteroid, seven showed abnormal Waugh-Ruddick tests. However, of six patients with abnormal heparin tolerance, only two had accelerated Waugh-Ruddick tests, and only two had accelerated clotting in lusteroid. Of five patients having abnormally short whole plasma prothrombin times, four showed abnormal Waugh-Ruddick tests, but only two had

accelerated clotting in lusteroid. The only patient among the 29 tested who developed venous thrombosis had normal values for all tests. This patient was a 42 year old colored man, who had had a hernia repaired eight days prior to the thrombosis. The clotting tests were performed five days prior to the occurrence of the thrombosis.

In order to ascertain how long the state of accelerated coagulation may persist after operation, the same series of six tests was repeated on seven of the 29 patients later in their postoperative courses. The results are shown in Table III. As may be seen in the table, evidence of hypercoagulability was found as late as 12 days after operation in some instances.

Our clotting times after 25 mg. of heparin were shorter than those obtained by Hagedorn and Barker (11), probably because our tests were performed at 37° C, whereas their tests were performed at room temperature, which is generally much lower. In order to study the effect of temperature upon the results obtained in this test, we

TABLE III
Persistence of hypercoagulability following operation

Operation	P. O. day	Pro. time whole	Pro. time 12.5%	Lee-White saline	Lust.	Heparin tol.	Waugh-Ruddick
		<i>sec.</i>	<i>sec.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
1. Hernia	2	15	30	9½	31½	17½*	66
	12	12.5	28.5	17½	26	36	72
2. Hysterect.	3	12.5	30.5	9	27	22	52*
	11	11.5	23.5*	13	29	42	48*
3. Gunshot bowel	4	10.5*	27	15	16*	49	64
	12	14	33.5	15½	24	22*	64
4. Hernia	2	12.5	31.5	27	32	45	82
	9	12.5	31	10	37	36	68
5. Perf. ulcer	6	11*	26	10	19	27	50*
	12	16.5	45	16	27	24*	58*
6. Append.	4	14	35	11½	30	28	54*
	11	12.5	35	14	29	31	66
7. Gunshot liver	2	12	30	12	25	48	60*
	10	16	34.5	10	26	62†	58*

* Accelerated value

† Abnormal hypocoagulability

ran clotting times on samples of blood drawn 10 minutes after giving 25 mg. of heparin intravenously to each of 10 patients. One part of the sample was tested at 22.5°, the average winter room temperature, and the other portion was done at 37°. As may be seen in Table IV, the clotting time was appreciably longer at 22.5° in all instances save two. The average clotting time at 22.5° was 26.5 minutes, and at 37°, 13.4 minutes.

In order to study the effect of test tube size upon the results obtained in doing the Lee-White clotting time, this test was done in glass tubes of

two different sizes, one 8 mm. by 75 mm. and the other, 13 mm. by 100 mm., using identical samples of blood. As shown in Table V, this procedure was performed in 14 instances. It may be seen that the clotting times were longer in the small tubes in nine instances; the clotting times were equal in one case; in the four remaining instances the clotting times were longer in the large tubes. Both Kadish (1) and Quick (16) found clotting times to be longer in large than in small glass tubes.

Whether or not the veins selected in performing the heparin tolerance test affected the result was also studied. In 14 instances, the one tube Lee-

TABLE IV
The effect of temperature on the heparin tolerance test *

Before heparin min.	10 min. after heparin 37° C min.	10 min. after heparin 22.5° C min.
1. 7½	22	38
2. 9	11	52
3. —	5	12
4. 7	12	27½
5. 9	17	42
6. 6	18	18
7. 7	16	20½
8. —	10	14
9. —	12½	12
10. —	11	29

* Blood specimens were drawn 10 minutes after 25 mg. heparin intravenously, and were divided into two portions. Most of these are abnormal cases, i.e., postoperative cases.

TABLE V
The effect of tube size on the three tube Lee-White clotting test

	Tube 8 mm. by 75 mm. min.	Tube 13 mm. by 100 mm. min.
1	12	10
2	13	10
3	15	13
4	11	9
5	11	11
6	12	6½
7	16	12
8	13	10½
9	10	11½
10	21	11
11	10	11
12	12	13½
13	10	10½
14	11	10½

White test was performed 10 minutes after injecting 25 mg. of heparin intravenously. Blood was drawn almost simultaneously from the vein into which the heparin was given, and from the vein of the opposite arm. As seen in Table VI, the results were the same or nearly so in seven instances. In six cases, the clotting times were considerably longer on blood drawn from the vein which had received the heparin. In one instance, the blood drawn from the vein receiving the heparin did not clot after 12 hours. In one case, the clotting time was significantly shorter on blood taken from the vein receiving the heparin. This finding would seem to imply that in some instances there is retained in the vein injected with a foreign substance a larger proportion of substance than is found in the general circulation.

TABLE VI
*The effect of veins used upon the results
of the heparin tolerance test*

	Before heparin	10 min. after heparin same vein	10 min. after heparin op- posite arm
	<i>min.</i>		<i>min.</i>
1	6	39½ min.	12
2	6½	12 hours plus	10
3	8½	14 min.	14
4	7	8*	9*
5	7	73†	50†
6	7	13½	12
7	5½	22½	24
8	5	12	13
9	5	21	12
10	—	7*	5*
11	7	10*	16*
12	—	13	10
13	—	20	12½
14	—	10	11

* Acceleration.

† 50 mg. heparin given instead of 25 mg.

Note: Each patient received 25 mg. heparin intravenously. After 10 minutes, one tube Lee-White clotting times were run on blood drawn almost simultaneously from the vein receiving the heparin and from the vein of the opposite arm.

DISCUSSION

The clotting mechanism in postoperative patients was studied by Potts and Pearl (20), Dawbarn, Earlham, and Evans (21), and by Shapiro Sherwin, and Gordimer (22). These investigators found that the platelet count tended to fall during the first three days postoperatively, but that it began to rise on the sixth postoperative day, and reached a peak on the tenth to 14th postoperative

day. They found little change in the clotting time as determined by the usual methods.

The abnormalities in the clotting mechanism found by us do not parallel the changes in the platelet count found by the above investigators; in general we found abnormalities in clotting earlier in the postoperative course in this study. Not only were the manifestations of derangements in the clotting mechanism found earlier here, but the change seemed to be greater earlier in the postoperative course than in the 10 to 14 day postoperative period when the maximum changes in the platelet count were found. However, as seen in Table III some cases still showed acceleration of clotting as long as 12 days after operation.

The finding of accelerated coagulation as judged by the Waugh-Ruddick test in 19 of the 29 postoperative patients is in keeping with the findings of Waugh and Ruddick (9), whose study of postoperative patients revealed accelerated coagulation 24 hours after operation; this acceleration was present seven days following operation. Our finding of acceleration of coagulation in two-thirds of 29 postoperative patients, a finding which persisted at least 12 days after operation in some cases, may be considered in regards to possible routine postoperative anticoagulant therapy.

The Waugh-Ruddick test seemed most sensitive of the tests employed in detecting the effect of operative trauma. In this connection, it is of interest to note that Flinn (23), following the administration of digitalis in man, found acceleration of coagulation as shown by the Waugh-Ruddick test, whereas the Lee-White test and the prothrombin time on whole and dilute plasma showed no change. The extreme sensitivity of the Waugh-Ruddick test is shown by its acceleration in response to such procedures as operative trauma and digitalization; this would seem to detract from its value in the prediction or diagnosis of venous thrombosis. The non-specificity of this test is further illustrated by the fact that Waugh and Ruddick found that bed rest alone caused acceleration of coagulation (9).

It is of interest to note the lack of correlation between the Waugh-Ruddick test and the heparin tolerance test, since heparin is used to prolong the coagulation of blood in both instances, in the former *in vitro* and in the latter *in vivo*. In partial explanation of this finding, the statements of

Best and Jaques (24) may be cited. These writers state that if there is mixture of the same amounts of heparin and blood *in vivo* and *in vitro*, the clotting time will be longer *in vivo* if moderate amounts of heparin are used. Best and Jaques also state that the renal threshold for heparin is quite low, and that there is rapid excretion of heparin into the urine if large doses are given intravenously. Further, even if heparin is not excreted into the urine, it disappears rapidly from the blood. Thus, variation in the rate of heparin destruction and heparin excretion have to be reckoned with in performing the heparin tolerance test. Since these factors are not operative in the Waugh and Ruddick test, the failure of the two tests to correlate may be explained.

The lack of correlation among all the tests might be explained in part by the onset of anxiety during the period of the test. DeTakats (25) has mentioned adrenal stimulation, such as by anxiety or fear, as a cause of increased coagulability of the blood. In a few cases we have found marked acceleration of coagulation in lusteroid tubes and marked increase of heparin tolerance when the patients were on the verge of an anxiety attack or delirium tremens. Other factors, such as type of anesthesia, the presence of an occult infection, or the administration of barbiturates might explain the variations in clotting from patient to patient in the postoperative group. However, since all six tests were done within a space of two hours on each postoperative patient, these last factors could not very well explain the lack of correlation in the individual patient; *i.e.*, these factors do not explain why some tests were normal and others abnormal in the identical patient at virtually the same time.

The prothrombin time of dilute plasma was less often abnormal than that of whole plasma. This was especially notable in the three thrombophlebitic patients, where the dilute plasma prothrombin time was normal in every instance, yet the whole plasma prothrombin time was shortened in all three. This finding is similar to that of Mahoney and Sandrock (26), who found a greater increase in prothrombin activity in their postoperative thrombophlebitis patients as shown by the test on whole plasma as compared to dilute plasma. Hurn, Barker, and Mann (27) also found that the prothrombin time of dilute plasma gave no more information as to hypercoagulability than the test as run on whole plasma. Tuft

and Rosenfield (28) concluded that accelerated dilute plasma prothrombin determinations could not be used for prediction or diagnosis of thromboembolic disease. In contrast, Shapiro (13, 14, 22) and Brambel and Loker (12) found the dilute plasma prothrombin time more sensitive to the state of hypercoagulability than the whole plasma prothrombin time.

Mahoney and Sandrock (26) found in their 58 normal postoperative patients a prolongation of the prothrombin time during the first three days following surgery. They found acceleration during this period only in those patients who subsequently developed thrombophlebitis. In our group, of 13 patients studied during the first three days postoperatively, none showed the prolongation of the whole plasma prothrombin time described by Mahoney and Sandrock (26). Further, the mean whole plasma prothrombin time in these 13 postoperative patients was 12.8 seconds, as compared with a mean of 13.5 seconds for the 28 controls. Among the 28 controls, the standard deviation for the whole plasma prothrombin time was 0.85 seconds. Among the 13 postoperative patients, the standard deviation for the whole plasma prothrombin time was 1.45 seconds. The standard error of the difference of the two means of 12.8 and 13.5 seconds was found to be 0.471 seconds. The observed difference was 0.70 seconds. This is not a significant difference.

Only one patient of this group of 29 postoperative patients developed thrombophlebitis after operation. The demonstration of frequent abnormalities in the six tests in so many of the 28 patients who did not subsequently develop thrombophlebitis would seem to indicate that none of these tests could be used to predict which patient is going to develop venous thrombosis following an operation. This statement is given weight by the fact that the only patient who did develop clinical thrombophlebitis showed no abnormality in any of the six tests which had been run five days previously.

It is thought worthy of comment that only one of the 27 patients having congestive heart failure showed an acceleration of coagulation in lusteroid tubes, and only two as measured by the three tube Lee-White test. This finding may be compared with that of Cotlove and Vorzimer (29) who found no shortening of the whole or dilute plasma pro-

thrombin time in 20 patients with congestive heart failure. It is also of interest to note that 25 of our 27 patients were receiving digitalis therapy in maintenance doses, yet little evidence of acceleration of clotting was found. Digitalis has been frequently mentioned as an accelerator of clotting (7, 25, 30). Ogura, Fetter, Blankenhorn, and Glueck (19) found some evidence of acceleration of coagulation in their patients as they were being digitalized, but found that this acceleration later disappeared in the majority, even though maintenance digitalis therapy was continued. Cotlove and Vorzimer (29) found that the administration of digitalis did not shorten the prothrombin time as determined on whole plasma or dilute plasma. Moses (31) found no change in heparin tolerance after the exhibition of digitalis. Flinn (23) found the Waugh-Ruddick test to show acceleration of coagulation after the administration of digitalis, but no acceleration was shown by the Lee-White test or by the prothrombin time on whole or dilute plasma.

SUMMARY

Twenty-nine postoperative patients were studied for evidence of accelerated coagulation. Nineteen showed acceleration by the Waugh-Ruddick test; ten showed acceleration in lusteroid tubes; six showed increased heparin tolerance; five showed shortening of the whole plasma prothrombin time; three showed shortening of the 12.5% plasma prothrombin time; three showed an acceleration by the modified Lee-White test.

The same tests were repeated on seven of these patients later in their postoperative courses and some showed persistence of hypercoagulability on the 12th postoperative day. The different tests did not correlate. The only patient who developed subsequent thrombophlebitis was normal to all tests. Three other patients with thrombophlebitis showed acceleration of coagulation in lusteroid tubes and shortening of the whole plasma prothrombin time. They were normal with regard to dilute plasma prothrombin time and the Waugh-Ruddick test. Of 27 patients with congestive heart failure, 25 of whom were receiving digitalis, only one showed acceleration of coagulation in lusteroid tubes, and two showed acceleration in the Lee-White test. Fourteen patients were given 25 mg.

of heparin intravenously. Ten minutes later, blood samples were drawn almost simultaneously from the arm vein into which the heparin had been injected, and from a vein in the opposite arm. In six of these 14 patients, the clotting time was significantly longer in the blood sample taken from the vein receiving the heparin when compared to the blood obtained from the opposite arm.

CONCLUSIONS

1. Hypercoagulability of the blood is a frequent finding in the postoperative state, and may persist as long as 12 days after operation.

2. The correlation among the Waugh-Ruddick test, prothrombin time on whole plasma, prothrombin time on 12.5% plasma, lusteroid tube clotting time, and heparin tolerance test is poor, suggesting that they are concerned with different factors involved in the clotting process.

3. We could not predict which postoperative patients were going to develop venous thrombosis.

4. When performing the heparin tolerance test and similar tests in which a substance is injected intravenously, and a sample of blood is later drawn for analysis, it is advisable to draw the blood from the vein of the opposite arm. The vein into which the substance is injected probably retains a larger proportion of that substance than is found in the general circulation.

5. Of the tests studied, the Waugh-Ruddick heparin retarded coagulation test appears the most sensitive. Its lack of specificity, however, would seem to indicate that it is of no value in predicting or diagnosing thrombophlebitis.

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STUDIES OF LUNG VOLUMES AND INTRAPULMONARY MIXING: NOTES ON OPEN-CIRCUIT METHODS, INCLUDING USE OF A NEW PIVOTED TYPE GASOMETER FOR LUNG CLEARANCE STUDIES

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INTRODUCTION

The measurement of lung volume by an open-circuit method (1, 2) is usually based on the redistribution of nitrogen contained in the lungs in such a manner that most of this gas is expelled and measured. Physically, this principle requires only the collection of the exhaled nitrogen in some diluent such as oxygen and the measurement of total volume and nitrogen concentration; the quantity of nitrogen involved can then be computed. In the usual procedure the subject breathes oxygen for a set period, the total expired air being collected in a gasometer; then the connecting tubes are washed with more oxygen and the final volume and nitrogen concentration in the gasometer are measured.

Such a method could be extended to the study of the nitrogen clearance process. The physical conditions which must be satisfied, however, are much more exacting than those which apply to measurement of lung volume. It becomes necessary to determine accurately (1) respiratory volume at various instants during the clearance process and (2) the total amounts of nitrogen in the external system at these instants. These requirements demand modification of existing open-circuit methods in the following particulars: *A.* It is necessary to avoid continuous flow of oxygen by-passing the experimental subject. Only by using a true demand system of oxygen supply can it be assured that all the gas collected has been involved in the tidal movement. *B.* It is necessary either to measure separate points on the clearance curve by doing several experiments, or to arrange for serial sampling of the expired gas, with simultaneous measurement of volume. The

latter expedient, which is preferable, implies in turn (1) the presence in the gasometer of some device to ensure rapid mixing of the gases introduced and (2) the elimination, as far as may be feasible, of tubes connecting the subject with the gasometer. If the latter condition is not satisfied, part of the gas exhaled in any breath will not reach the gasometer until the succeeding breath.

During experimental work described elsewhere (3, 4) some 30 modifications of the open-circuit method have been used in the measurement of lung clearance curves. It will perhaps be of value, in view of this extensive experience, to indicate the ways of meeting the foregoing technical requirements which have proved most reliable. This paper deals solely with the physical problems of measuring the volume and composition of expired gas and neither with physiologic sources of error nor with the interpretation of the data. Two distinct procedures, the "end-point sampling" method and the "serial sampling" method, will be described. The latter is greatly to be preferred, but it involves the use of special equipment.

THE END-POINT SAMPLING METHOD

Experimental arrangement. Figure 1 represents the arrangement which has been found most satisfactory. Oxygen is supplied from high pressure tanks after reduction to 100 pounds per square inch. This serves as the input line for a Pioneer dilutor-demand oxygen regulator, type AN6004-1, which has been modified by removing the dilutor cap and handle and the aneroid unit and sealing the opening with a gasket and bakelite plate. Thus the valve supplies only pure tank oxygen.² Since the operating suction is appreci-

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² We are indebted to the National Bureau of Standards and to Lieutenant Commander Allison of the Naval Bureau of Aeronautics for this regulator.

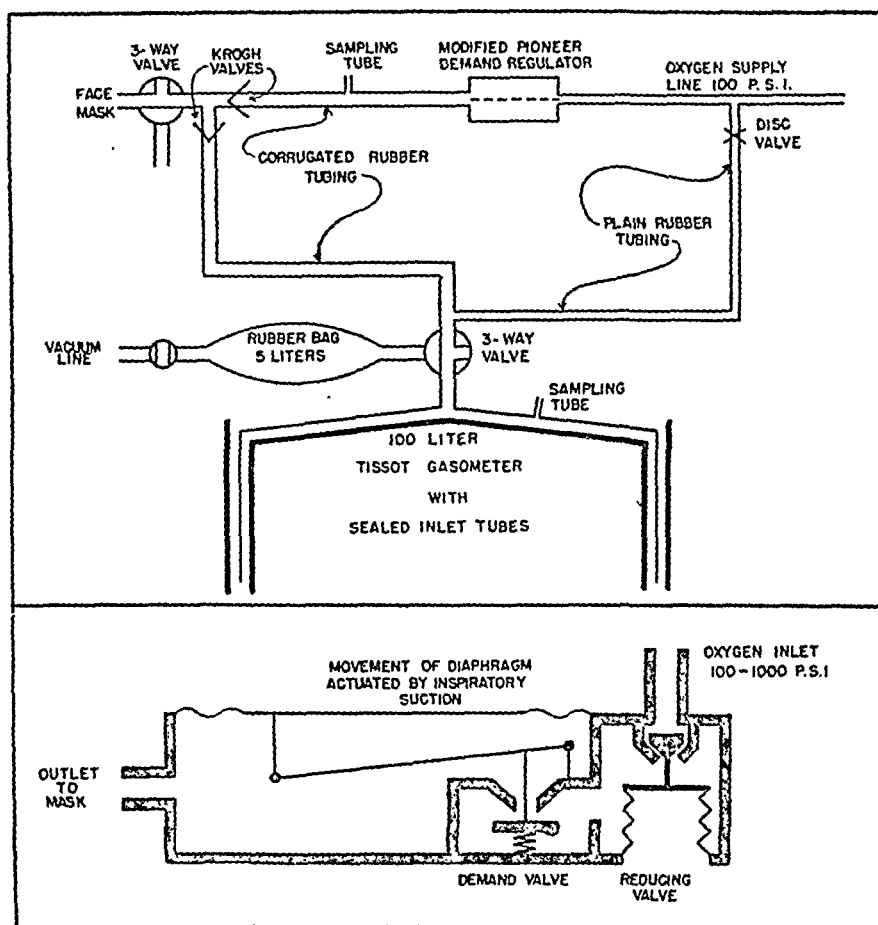


FIG. 1. THE END-POINT SAMPLING METHOD

Above: Open-circuit assembly for collecting nitrogen expelled during breathing of oxygen. *Below:* Diagram (schematic) of modified oxygen demand regulator.

able the regulator is loaded with a 15-gm. weight placed on the large diaphragm indicated in the lower portion of Figure 1.² The output side of the demand regulator is connected by large corrugated rubber tubing to a Krogh valve, to a three-way hand-operated valve of large bore and finally to the face mask worn by the subject. At a point slightly distal to the three-way valve, the inspiratory line communicates with the tube through which the expired gases pass, by way of a second Krogh valve, to the gasometer bell. The gasometer is of the Tissot type, of 100-liter capacity, modified by sealing the inlet tubes at their point of entry into the main chamber. This appreciably reduces the dead space. Welded to the roof of

the gasometer bell is a second manual three-way valve of large bore with a side-arm connected to a 5-liter rubber bag and vacuum line. The bag is used at the end of each experiment for mixing the collected expired gas in the gasometer, by being successively filled and emptied. The wire carrying the counterweight of the gasometer is supplied with a pen which indicates volume on a moving drum, not shown in Figure 1, during the collection of expired gas. The by-pass from the high-pressure oxygen supply to the gasometer indicated in the diagram serves for initial washing of the apparatus.

Attention may be drawn to (1) provision of a sampling tube for checking the composition of inspired gas, and (2) the valve assembly, consisting of two Krogh valves welded into a compact unit.

Analytic method. A modified Haldane method of gas analysis was used, the nitrogen residue being measured as the volume increment brought

² Evidence has been obtained that a certain amount of by-passing occurred in some of the experiments reported in the following papers as a result of failure of the valve to close completely when operated by persons with certain types of respiratory pattern. This source of error has now been obviated.

about after mixing with a larger measured volume of stored nitrogen. Duplicate analyses are usually reproducible to within 0.001 (0.10 per cent nitrogen).

The accuracy with which a given amount of nitrogen can be estimated after dilution with oxygen was investigated by progressively diluting a known amount of air (measured by displacement of water) in a gasometer of known dead space with measured amounts of oxygen and comparing the measured decreases of nitrogen concentration with those calculated from the volumes mixed (Table I). It seems fair to conclude from the final column of Table I that at no point in a clearance curve is the error in the total amount of nitrogen measured likely to exceed 100 ml. from this particular source of error; usually it will be much less. The accuracy seems to be

greatest when the nitrogen fraction in the gasometer is 1 to 3 per cent.

Accuracy of volume measurements. In the 100-liter Tissot gasometer, a movement of 1 cm. indicates a change of volume of about 2 liters. The scale can be read to about 0.25 mm., or 50 ml. This is adequate for most purposes provided it can be proved possible to determine the conditions of temperature and humidity to which the measured value applies. It has been found in repeated tests that the volume of expired air received by the gasometer does not undergo any observable change over a period of several minutes. Presumably, the gas leaving the subject at 37° C and saturated with water vapor is almost immediately cooled to the temperature of the gasometer and the corresponding pressure of saturated aqueous vapor is established. It follows

TABLE I
*Gasometric estimation of nitrogen diluted with varying quantities of oxygen **

(1) Volume of air originally in gasometer	(2) Nitrogen re- moved in samples	(3) Corrected vol- ume of air present (V _{air})	(4) Total volume of gas in gasometer (D + V)	(5) Measured N ₂ fraction in gasometer (fN''')	(6) Calculated volume of air in gasometer	(7) Differences between analytic and volumetric volumes of air	
cm. ³	cm. ³	cm. ³	cm. ³		cm. ³	cm. ³	per cent of (3)
1,112	0	1,112	13,640	0.0697	1,172	+ 60	+ 5.4
	22	1,090	32,800	0.0290	1,127	+ 37	+ 3.4
	9	1,081	73,440	0.0131	1,042	- 39	- 3.6
	4	1,077	93,620	0.0106	1,032	- 45	- 4.2
2,104	0	2,104	12,530	0.1399	2,192	+ 88	+ 4.2
	44	2,060	32,800	0.0522	2,091	+ 31	+ 1.5
	17	2,043	73,360	0.0248	2,129	+ 86	+ 4.2
	8	2,035	93,620	0.0198	2,124	+ 89	+ 4.4
1,115	0	1,115	12,530	0.0744	1,151	+ 36	+ 3.2
	23	1,092	32,900	0.0285	1,109	+ 17	+ 1.6
	9	1,083	73,360	0.0133	1,060	- 23	- 2.1
	4	1,079	93,620	0.0097	925	- 154	- 14.3
2,100	0	2,100	12,530	0.1379	2,160	+ 60	+ 2.9
	44	2,056	32,800	0.0520	2,083	+ 27	+ 1.3
	17	2,039	73,440	0.0245	2,104	+ 65	+ 3.2
	8	2,031	93,620	0.0190	2,029	- 2	- 0.1

* Procedure: The gasometer dead space is originally filled with tank oxygen. A known volume of air is admitted to the gasometer by displacement with a weighed amount of water in an Erlenmeyer flask (column 1). A known volume of oxygen is then added, the gasometer volume is read (column 4) and a sample is removed for analysis (column 5). Then further oxygen is added and the procedure is repeated, the original volume of air in the gasometer being corrected at each stage for the amount removed in the sample (column 2). The corrected volume of air (column 3) can be compared with that calculated from the nitrogen analyses (column 6).

$$V_{\text{air}} = \frac{(V + D)(fN''' - fN)}{0.791 - fN}$$

where D = dead space of gasometer = 2,390 cm.³,
V = volume of gas in gasometer,
V_{air} = corrected volume of air in gasometer,
fN = nitrogen fraction in tank oxygen (usually about 0.003),
fN''' = nitrogen fraction in gasometer.

also that no measurable errors are introduced by transfer of gas to or from the water in the gasometer.

Gasometer dead space. Even when measures were taken to reduce the dead space of the Tissot gasometer, the value remained in excess of 2 liters. This is undesirably large and its presence has reduced the accuracy of our measurements in the early part of the lung clearance curve. A more serious drawback is the fact that this dead space is less constant than might be desired, since the position of the gasometer bell when the gasometer is "empty" is variable. This is difficult to eradicate in a gasometer of the Tissot type.

Procedure in end-point sampling method. For some hours before the experiment is started, the entire system is washed with a slow stream of cylinder oxygen. Analyses of the gas in various parts of the system are made. The gasometer is then emptied, the rubber bag is evacuated, and the three-way valve to the gasometer is oriented as shown in Figure 1. The subject is connected to the apparatus with a suitable face mask, with the three-way valve so turned that he is able to breathe room air. After doing this for a short time, he rapidly turns the three-way valve at the end of a normal expiration so as to be brought into connection with the oxygen supply system and the gasometer. He breathes oxygen for the desired number of breaths—ten for example—and at the end of the tenth exhalation, turns his three-way valve back to the original position. A volume reading is taken on the gasometer and the connecting tubes in the expiratory line are flushed with 10 liters of oxygen. The three-way valve on the gasometer is turned so that the rubber bag fills itself from the gasometer contents; it is then emptied by hand and filled again, and the cycle is repeated ten times. The gas is then deemed adequately mixed and samples are collected over mercury in a 100-ml. tube. In order to get further points on the clearance curve, the system is again washed with oxygen and after a suitable intervening period of at least 30 minutes, during which the subject breathes air, the entire procedure is repeated.

THE SERIAL SAMPLING OF COLLECTED EXPIRED AIR

The study of lung clearance curves by the method described in the preceding section is at

best a laborious procedure. In any extended investigation, it is desirable to develop methods for detailed recording of a single clearance process. Such a method, which would involve taking a series of instantaneous samples of mixed expired gas, would have further advantages, including the reduction of errors resulting from variations in the initial lung volume. For this and other reasons, methods for serial sampling have been devised.

Four new requirements must receive attention in connection with serial sampling: 1. Provision for rapid mixing of gases must be made, so that any sample is truly representative of the entire contents of the gasometer. 2. The volume of the tubes connecting the subject with the gasometer must be reduced because it represents a volume of expired air which, at every exhalation, fails to enter the gasometer and is thus unable to contribute to the composition of the mixed expired gas. 3. An improved sampling procedure is required, since it is necessary to take samples rapidly and at frequent intervals. 4. The gasometer dead space should be reduced.

A NEW GASOMETER ASSEMBLY FOR LUNG CLEARANCE STUDIES BY SERIAL SAMPLING

The gasometer. A new gasometer with associated equipment has been designed with the foregoing considerations in mind, together with the following additional points which appeared important: (a) low inertia of moving parts, (b) convenient and accurate recording of collected gas volume and (c) capacity large enough to hold most of the air expired during lung clearance but small enough for adequate accuracy in the recording of volume and also small enough to make it convenient to conduct a clearance measurement in two parts, since the final portion of the clearance curve can be more accurately determined in this manner than would be possible in a single protracted period of collection.

The plans for the new gasometer were placed in the hands of the Waters Conley Company, Rochester, Minnesota, which was responsible for mechanical details and for the construction of the apparatus.

A gasometer of the pivoted type (5, 6) appeared to offer decisive advantages, the form being such as to permit close approach of the subject, so that the dead space of the connecting tube can

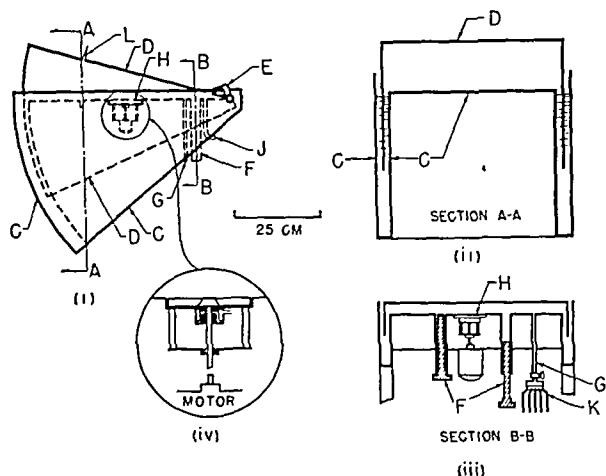


FIG. 2. PIVOTED TYPE GASOMETER

Side view and sections showing positions of gas agitator, inlet tubes and sampling device. See text.

be reduced to a few cubic centimeters. Such an apparatus also appeared amenable to fine mechanical construction, so that the internal dead space could be much reduced. Moreover, since the bell is not freely suspended but can be accurately and rigidly pivoted, this dead space should be highly reproducible. Added to these advantages is the ease with which the necessary mechanical linkages can be made for recording gas volume.

The layout of the apparatus can be gathered from Figures 2 and 3. The gasometer bell consists of a wedge-shaped shell of about 60 liters capacity (Figure 2, D) made of sheet aluminum (roof 3 mm. thick; side walls 0.75 mm.) pivoted at one end (Figures 2 and 3, E) with "New Departure" precision ball bearings. It articulates in a water-filled well of the form indicated by C (Figure 2), which thus takes the form of a rectangular moat bounding a rectangular floor of sheet brass (Figure 2 [ii]). When the bell and the trough articulate, the volume of gas between them is completely enclosed by a water seal. The floor is pierced by several holes. These are conveniently placed near the axle of the machine. The centrally situated tube, J (Figure 2), of internal diameter 1.3 cm., serves for the introduction of expired gas. A narrow sampling tube, G, of internal diameter 0.3 cm., passes to the lucite distributor (see also page 684). Two wide apertures, F, are included, so as to make the apparatus rapidly and conveniently adaptable for use in open-circuit or closed-circuit measurements of

metabolic rate. Normally, these tubes are kept closed by brass plugs which pass in as far as the level of the floor of the gasometer chamber, thus eliminating additional dead space. Finally, an opening, H (Figure 2), in the center of the floor carries the driving shaft of a circulating fan set in a depression 10 cm. in diameter and 0.6 cm. deep. This fan, of centrifugal type, and its water-sealed bearing are shown in more detail in Figure 2 (iv).

The two recording systems are shown in Figure 3. For photographic recording, the horizontal axis about which the gasometer bell moves is linked by means of a flexible cable to a vertical axle, U, supported by two precision ball bearings and provided with a mirror (Figure 3 [iii]). For mechanical recording, a wire attached to the lowest peripheral point, M, of the gasometer bell passes over a fixed pulley, N, to the ink-writer pulley, P, and thence to a counterweight, O, which is adjusted to keep the gasometer bell very nearly balanced in all positions. The method of attachment of the recording wire to the gasometer bell is of some importance, since the effective point of attachment is determined by the tangent from the pulley, N, to the circular arc defined by the end of the gasometer bell. The point of attachment is therefore effectively constant for all positions of the bell, so that a linear relation between volume and recorded deflection is assured. A system of gears introduces the possibility of amplifying the volume record in accordance with the following figures: for direct recording, 1 liter volume change produces 6.5 mm. deflection; for amplified

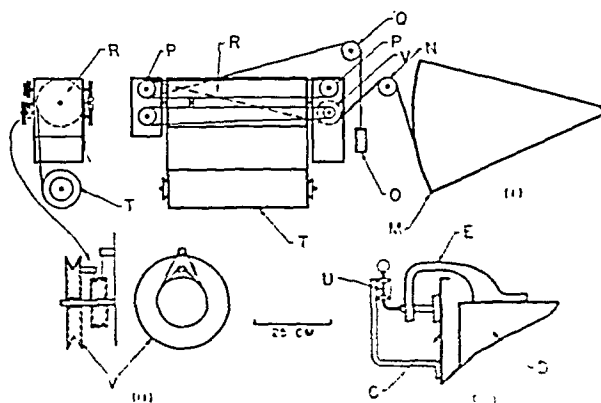


FIG. 3. DETAILS OF RECORDING MECHANISMS OF PIVOTED TYPE GASOMETER
(SEE TEXT.)

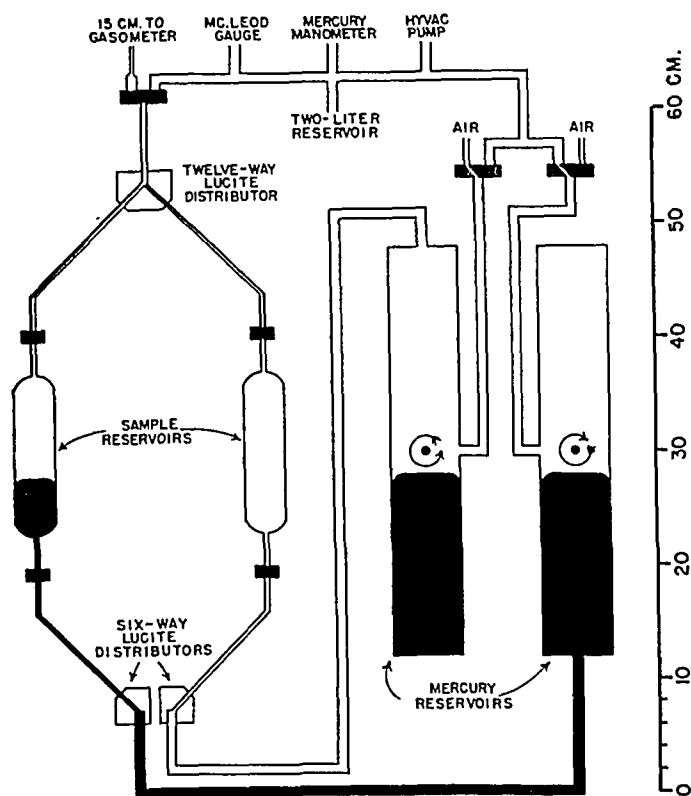


FIG. 4. EXPERIMENTAL ARRANGEMENT USED FOR COLLECTING SERIAL SAMPLES OF EXPIRED AIR IN EVACUATED SAMPLE TUBES

Operation described in text.

recording, the deflection is 25 mm. Furthermore, a clutch system operated by the pulley, V, drawn in detail in Figure 3 (ii), enables the lower pen to operate as an indicator of total ventilation. This is not used in lung clearance measurements, but is useful in closed-circuit metabolism studies.

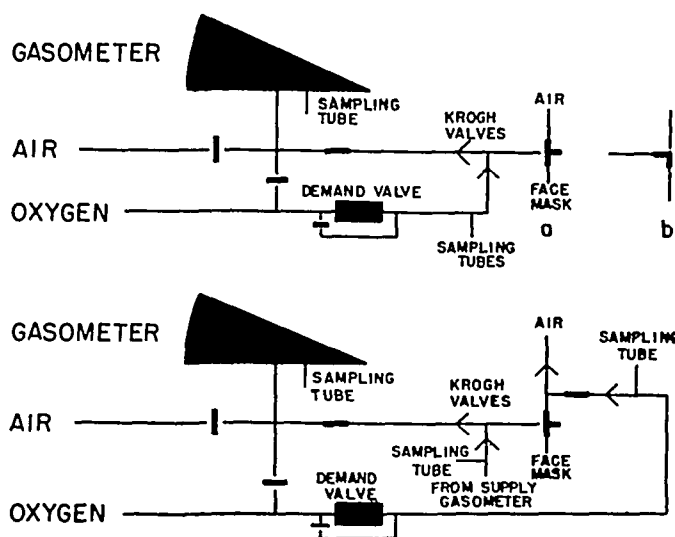
The entire apparatus is mounted with adjustable clamps on two steel pillars 7 cm. in diameter attached to a wheel base, vertical adjustment being facilitated by counterweights.

Sampling procedure. Samples from the gasometer are collected over mercury in evacuated 100-ml. Pyrex tubes. Twelve such tubes are connected through a lucite distributor to the sample tube of the gasometer (Figure 4). The tubes are evacuated by a Cenco pump before the experiment is commenced and the rest of the system, consisting of the dead space of the distributor, the 2-liter reservoir, and various mercury traps and connecting tubes, is kept evacuated throughout the experiment. When a sample is to be taken, the entire distributor is filled with gas from the gasometer; then the appropriate sample tube is opened and closed. Immediately thereafter, the distribu-

tor is once more connected to the vacuum line and is evacuated in time for the next sample. At the end of the experiment, mercury is admitted to the sample tubes so as to bring the samples to atmospheric pressure and reduce the danger of inboard leaks.

The mercury reservoirs shown in Figure 4 were necessary in order to free the mercury from air and to obviate the danger of contamination by inward leakage of air through flexible connecting tubes. It was found that both rubber and plastic tubing are permeable to air, so that without special precautions, large analytic errors were caused by air leaking into the mercury and thus being transferred to the gas samples. The mercury reservoirs were designed in such a way that during the preliminary removal of air from the system all flexible connecting tubes could be emptied and evacuated. The mercury itself could be agitated by inverting

1. NITROGEN ELIMINATION



2. NITROGEN RETENTION

FIG. 5. SERIAL SAMPLING ARRANGEMENT USED IN STUDY OF ELIMINATION AND RETENTION OF NITROGEN BY THE LUNG

(1) By collecting expired gas during the breathing of pure oxygen, (2) By collecting expired gas during breathing of air following prolonged inhalation of oxygen. Connecting tubes are represented by thin continuous lines. Thick lines indicate the presence of hand-operated valves. Arrows represent Krogh valves. The gasometer is a pivoted type apparatus of 60-liter capacity provided with a fan to accelerate mixing of gas collected. Serial samples of total expired gas are taken through the sampling tube in the floor of the gasometer, using the distributor shown in Figure 4.

the reservoirs by rotation about a central axle as indicated by the arrows in Figure 4. As soon as the reservoirs were restored to their original position and mercury was again introduced into the flexible tubing, it was possible to admit air to the reservoirs and to maintain the mercury at or above atmospheric pressure. The entire system, including the lucite distributor, was tested routinely for leakage by reducing the pressure to about 0.1 mm. of mercury and observing its constancy with a McLeod gauge (Stokes, type 276 AA).

Dead space. The volume of gas in the gasometer and fixed connecting tubes when the moving shell is completely collapsed was measured both by filling this volume with tank oxygen and diluting with a known amount of air or nitrogen, and also by filling with air and diluting with tank oxygen. Concordant and reproducible values were obtained. The volumes of the conducting passages distal to the Krogh valve assembly and of the connections from the Krogh valve assembly to the face mask (Figure 5) were determined from their physical dimensions. The dead space of the face mask was estimated in several persons by filling it with water and measuring the amount of water remaining after the mask had been placed over the face. As it was not feasible to perform this type of measurement on every subject, a single average value was used throughout. The three dead-space volumes, measured in milliliters, were (1) "internal" dead space of gasometer, d , 544 ml., with which may be contrasted the value of about 2,000 ml. in the modified Tissot gasometer used in the "end-point sampling" experiments; (2) "external" gasometer dead space, D , 85 ml.; (3) external "respiratory" dead space, d_c , 150 ml. (volume of mask and connecting tubes as far as Krogh valve assembly).

Mixing of expired gas in gasometer. During the experiments reported elsewhere (3, 4) the fan used for mixing the expired gases was operated by a small motor rated at 3,600 r.p.m. This fan was later found to be erratic in performance and mixing was not sufficiently rapid. The motor was replaced by one rated at 7,000 r.p.m. connected through a Variac transformer, and the rate of mixing was measured as a function of the transformer setting. The sampling error attained an acceptably low value with an undetermined motor speed somewhat less than 7,000 r.p.m. The magnitude of

these sampling errors is discussed elsewhere (4).

Assembly; experimental procedure. The final assembly used in lung volume and lung clearance studies is shown in the upper portion of Figure 5, together with the slight modification (lower diagram) when nitrogen retention during the breathing of air is to be measured. The assembly is essentially similar to that used in the end-point sampling method except for the fact that the sampling tube is connected to the evacuated lucite distributor and multiple reservoir system already described. The sampling tube indicated in the oxygen line is for determining the composition of the supply oxygen from time to time and for establishing the absence of air leaks.

The procedure is also similar to that already described, save for changes necessitated by serial sampling. The sampling procedure involves counting of the number of breaths of oxygen, rapid manual operation of the stopcocks in the distributor assembly during the appropriate *inspiration*, and simultaneous operation of an electric signal which records the spirometer volume on the ink record at the instant of sampling. The technic needs to be rehearsed a few times.

SUMMARY

The extension of open-circuit methods of measuring lung volumes to a study of lung clearance rates requires certain physical modifications which are enumerated above (see also page 679). The necessary modifications have been embodied in two experimental assemblies and procedures referred to as "end-point sampling" and "serial sampling."

The assembly designed for end-point sampling experiments is composed of standard pieces of equipment, slightly modified. The method, although capable of considerable accuracy, is laborious.

The assembly designed for serial sampling requires a specially designed gasometer of small internal and external dead space, equipped with means for rapid mixing of the expired gases and with attachments for rapid sampling. With such an assembly, lung clearance curves can be obtained in about one-tenth of the time required by the end-point sampling method. A pivoted type gasometer designed and built for use in the serial sampling

apparatus, but also suitable for standard spirometric studies by open-circuit or closed-circuit methods, is described.

Experimental procedures are given for the study of pulmonary clearance curves by the two methods.

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OBSERVATIONS ON THE PROTEOLYTIC ACTIVITY *IN VITRO* AT NEUTRAL REACTION OF GASTRIC JUICE FROM PATIENTS WITH SPRUE¹

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Previous investigators (1) have demonstrated the presence in normal human gastric juice of a protease which is active at neutral reaction. Certain properties were exhibited which distinguished the enzyme from pepsin and trypsin. Subsequent studies (2-4) showed that the protease and the so-called intrinsic factor of Castle possessed certain identical properties: both are removed by adsorption with Lloyd's reagent, both are unable to penetrate a semi-permeable membrane, they are not destroyed by Berkefeld filtration or exposure to alkali, but are destroyed by exposure to heat and are inhibited by an environment more acid than pH 3.5. Furthermore, it was found that this protease activity was absent or greatly diminished in patients with Addisonian pernicious anemia, but was normal in patients with pernicious tapeworm anemia.

The pathogenesis of the macrocytic anemia in sprue has aroused much speculation. Because it seemed important to determine whether or not the intrinsic factor was contained in the gastric juice of this disease, and because the gastric protease which is active at neutral reaction may be identical with Castle's intrinsic factor, it was decided to measure gastric protease activity at neutral reaction in sprue.

METHODS

The method was similar to that previously described (1), except for modifications noted below. Gastric juice was collected free from bile after stimulation with histamine and promptly filtered through gauze. The juice was brought to pH 10 with sodium hydroxide and kept for 30 minutes at 40° C, after which it was brought to pH 7.4. To 50 ml. of such materials were added 50 ml. of a 1 per cent neutral casein solution, the mixture ad-

justed to pH 7.4 and 2 ml. of toluol added. The mixture was incubated at 37.5° C for 24 hours, the pH remaining constant. The procedures used for the formol titration of amino nitrogen and for the determination of total filtrable nitrogen were those previously described (1, 2). Samples of the digests were taken immediately, at four hours, and 24 hours for formol titration. At the same time intervals, samples were removed and analyzed for the total amount of nitrogen not precipitable by 10 per cent trichloroacetic acid. The amount of total filtrable nitrogen and of amino nitrogen produced in 24 hours was calculated from these determinations. Proteolytic activity was judged from increases in filtrable nitrogen in milligrams per 100 ml. digest. The absence of active trypsin was shown by the lack of a significant increase in amino nitrogen.

RESULTS

From a large number of experiments the following facts were confirmed:

(1). *The effect of incubation of normal human gastric juice with casein solution at pH 7.4.* The fasting contents from normal subjects were removed and discarded. Histamine phosphate 0.5 mgm. was injected intramuscularly and the gastric secretion collected during the next hour. Certain physical changes occurred when such gastric juice was incubated with an equal quantity of 1 per cent casein solution at pH 7.4. The digestion mixtures became chalky white. The trichloroacetic acid filtrates from these serial samples showed progressive increases in turbidity. Increases in the total nitrogen in the trichloroacetic acid filtrates occurred along with these physical changes.

Peptic activity is not manifest at neutral reactions, and above pH 8 pepsin is destroyed rapidly by OH ions. Furthermore, it has been shown (5) that exposure to alkali at pH 10 at 40° C for 30 minutes destroys from 70 to 80 per cent of trypsin in solution. There was no significant increase in amino nitrogen in our experiments. This suggests that trypsin and other erepsin-like enzymes of duodenal origin did not account for increase in

¹ Aided by grants from the John and Mary R. Markle Foundation, and the Duke University Research Fund.

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filtrable nitrogen. The results of ten experiments on normal controls, which agree with previous reports, are shown in Table I.

That infection may have an inhibitory effect is suggested from determinations made on gastric juice obtained from patients at the time of an acute respiratory illness and following convalescence. The results of two such experiments are included in Table I. This would indicate that the gastric protease is inhibited during an acute illness and that the inhibitory factor disappears following recovery.

(2). *The effect of incubation of human gastric juice, collected from sprue, with casein solution at pH 7.4.* Collections of gastric juice from sprue patients and treatment with alkali before mixing with casein for digestion were performed in a fashion similar to that used on normal controls. The results of 11 of these experiments on sprue are shown in Table II. The gastric juice obtained from cases of sprue in remission showed progressive increases in filtrable nitrogen which compared closely to results in normal controls. On the other hand, the gastric juice from cases of sprue in relapse, with characteristic diarrhea, meteorism, steatorrhea, weight loss, glossitis, and anemia, showed a greatly diminished production of total filtrable nitrogen. Also, it is noteworthy that there was no significant increase in amino nitro-

TABLE II

Effect of incubation with equal quantity of 1 per cent casein solution at 37.5° C and pH 7.4 of preparations of gastric juice

Experiment number	Preparation and reference to clinical observation	Increase in nitrogen in trichloroacetic acid filtrates (mgm. per 100 ml. digest)		Increase in amino nitrogen by formal titration (mgm. per 100 ml. digest)	
		4 hours	24 hours	4 hours	24 hours
30	Sprue-relapse-gastric juice after incubation pH 10, 40° C for 30 minutes	12.0	13.6	0.0	0.4
31a*		7.4	23.2	0.0	0.2
32		12.0	21.0	0.6	1.6
33		2.7	22.6	0.4	0.8
34		4.8	14.3	0.2	1.4
31b*	Sprue-remission-gastric juice, after incubation pH 10, 40° C for 30 minutes	40.5	47.6	0.0	0.4
35		49.7	59.0	0.0	1.4
36		21.4	55.0	0.0	0.7
37†		18.2	36.7	1.6	1.8
38		32.0	49.0	0.2	0.8
39		22.5	48.6	0.8	1.2

* Samples of gastric juice 31a and 31b were obtained from the same donor. Sample 31b was obtained five months later during remission.

† Case 37 had a-chlorhydria.

gen. Experiments 31a and 31b noted in Table II were performed on the same patient during a period of relapse and remission respectively. These data would indicate that the gastric protease is distinguishably less during the relapse stage.

DISCUSSION

In the majority of sprue patients, gastric secretion was obtained without difficulty in a one-hour period. During the stage of severe relapse there was usually hypochlorhydria and a marked reduction in rate of secretion and in proteolytic activity at neutral reaction. Such patients were observed again and tests repeated six months after treatment had produced remission, and they then showed a return to normal in all these functions. These data suggest that intrinsic factor activity may also be reduced in the gastric secretions of persons with sprue in relapse.

Attempts were made to do a biologic assay of the gastric secretions from these patients in relapse to determine the presence or absence of intrinsic factor. Our efforts to collect sufficient gastric juice for daily feeding experiments failed. However, previous investigators (6) have performed a biologic assay on gastric secretions from a case of sprue. Apparently, they demonstrated the absence of intrinsic factor from the gastric contents of a

TABLE I

Effect of incubation with equal quantity of 1 per cent casein solution at 37.5° C and pH 7.4 of preparations of gastric juice

Experiment number	Preparation and reference to clinical observation	Increase in nitrogen in trichloroacetic acid filtrates (mgm. per 100 ml. digest)		Increase in amino nitrogen by formal titration (mgm. per 100 ml. digest)	
		4 hours	24 hours	4 hours	24 hours
11	Control-normal gastric juice after incubation pH 10, 40° C for 30 minutes	37.5	59.3	1.6	1.8
12		14.5	44.5	0.3	2.0
13		31.0	42.5	0.8	1.0
14		36.4	54.7	0.4	0.7
15		32.8	48.0	0.2	0.6
16		20.3	34.6	0.5	0.8
17a*		4.2	21.0	0.0	0.1
17b*		35.2	42.0	0.7	1.4
18a†		7.4	9.2	0.0	0.0
18b†		28.8	36.0	0.6	0.8

* Samples of gastric juice 17a and 17b were from same donor. Sample 17a was collected during an episode of acute tonsillitis. Sample 17b was collected one month later.

† Samples 18a and 18b from same donor. Sample 18a collected during acute attack of sinusitis. Sample 18b collected three weeks later.

case of sprue in relapse by feeding daily collections of gastric juice mixed with beef muscle to a case of Addisonian pernicious anemia.

It has been stated (7) that the deficiency in sprue may come about in a number of ways, among which are (1) a deficiency of extrinsic factor, (2) a defect of gastric digestion resulting from lack of intrinsic factor, and (3) defective absorption from the intestinal tract.

The mal-absorption from the small intestine in patients during relapse can be demonstrated with great frequency and certainly is a major cause of the sprue syndrome. Defective absorption of fats, fat-soluble vitamins, calcium, and carbohydrates can be commonly demonstrated. Patients with severe diarrhea will also show faulty assimilation of protein.

Dietary histories on our sprue patients have not shown any correlation between the ingestion of meat products, or other sources of extrinsic factor, and the incidence of anemia. When fed adequate amounts of beef muscle, the patients in relapse failed to obtain a remission. Subsequently, they made a satisfactory response to injectable liver extract or folic acid.

The data presented in this paper support the concept that a defect in gastric digestion may be important to the occurrence of macrocytic anemia in sprue; though whether such a defect is primary or secondary to gastro-intestinal mal-absorption and chronic debility can not be ascertained. Data reported here show that acute infection can lower the value of the proteolytic activity at neutral reaction to a very low level.

The fact that the anemia of sprue is macrocytic and improved by liver does not mean that a primary deficiency of gastric origin is present. This is well shown by the pernicious tapeworm anemia.

It can only be said that the protease activity of the gastric juice is lessened in sprue in relapse and normal in remission.

CONCLUSIONS

1. When equal quantities of normal human gastric juice and 1 per cent casein solution are incubated at 37.5° C and pH 7.4, there is a progressive increase in filtrable nitrogenous substances. The proteolysis was not considered to result from pepsin because the activity was maximal at pH 7.4. Also, since there was no significant increase

in amino nitrogen within 24 hours, together with the persistent increase in total filtrable nitrogen after exposure to pH 10, it is considered that the proteolysis was not due to trypsin.

2. The proteolytic activity of the gastric enzyme at neutral reaction, is present to a normal degree in cases of sprue in remission. It is greatly diminished in cases of sprue in relapse. The absence of proteolytic activity in cases of pernicious anemia suggests that the deficiency of intrinsic factor so characteristic of pernicious anemia, may be present in sprue in relapse.

3. In two non-anemic normal subjects, it appears that infection inhibited this type of proteolysis to a considerable degree.

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SODIUM AND CHLORIDE DEPLETION IN ACUTE PORPHYRIA WITH REFERENCE TO THE STATUS OF ADRENAL CORTICAL FUNCTION

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INTRODUCTION

The fact that depletion of sodium and of chloride may occur in acute porphyria, in the absence of alkalosis due to vomiting, has recently been noted independently by Abrahams, Gavey and MacLagan (1) and by Linder (2). These authors suggested that a possible basis for this deficiency was that of adrenal cortical failure, but the response of their two patients to substitution therapy with cortical hormones does not appear to have given unequivocal evidence for this hypothesis.

The present paper presents an attempt to explore this question in further detail. The plan of investigation falls under two headings: firstly to study the function of the adrenal cortex during the height of an attack of acute porphyria and secondly to observe the response to desoxycorticosterone administration in greater detail. Prunty, Forsham and Thorn (3) have recently indicated that the elucidation of adrenal cortical function with respect to its relation to electrolyte metabolism is a complex matter, whereas the functional production of the 11-oxy-steroid group of hormones is more easily assessable by urine analysis and the use of the response to purified adrenocorticotrophic hormone. The severity of the illness of the patient under observation was considered a contraindication precluding the use of the deprivation test of Cutler, Power and Wilder (4).

METHODS

The patient (Case 1) was confined to bed during the first 80 days of observation. During the period of electrolyte study she was maintained on a light diet, kept as constant as possible, with the addition of 7 gm. of sodium chloride daily, the major portion of which was given orally as half normal saline. Urine was collected daily under toluene and analysed the day of completing the collection. The methods of analysis used were as follows. Sodium by the method of Butler and Tuthill (5);

chloride by the iodometric method of Van Slyke and Hiller (6); potassium by King's method (7); carbon dioxide combining power by the technique of Peters and Van Slyke (8). Total base in serum was estimated by electro dialysis (Malm [9]). "Neutral reducing steroids" in the urine were determined by the author's modification of the method of Talbot *et al.* (10),¹ and 17-ketosteroids by that of Callow, Callow and Emmens (11), using a correction equation for interfering chromogens. The adrenocorticotrophic hormone test for adrenal cortical reserve has been fully described by Forsham *et al.* (12). Total urine porphyrin was estimated by adsorption on a calcium phosphate precipitate followed by solution in hydrochloric acid as described by Sveinsson, Rimington and Barnes (13), the final assay of porphyrin being carried out in the fluorimeter.

OBSERVATIONS

Plasma electrolyte changes

The trend of the alterations found in the plasma electrolytes is indicated in Table I, and Figures 1 and 2. At the commencement of the period of observation the patient was receiving potassium citrate, 2.7 gm. per diem, on account of mild urinary symptoms. At this time (15th day) there was a moderate deficiency of sodium and of chloride, the values being 125 and 86 MEq. per l. respectively. The carbon dioxide combining power was normal (30.4 MEq. per l.). It will also be noted that there was a moderate elevation of blood pressure, a characteristic of this disease. On the 19th day potassium citrate was withdrawn and on the 20th day a six-day period of desoxycorticosterone acetate administration commenced. This was given in two doses per day of 5 mgm. each intramuscularly. On the fifth day of desoxycorticosterone administration the serum potassium had

¹ The determination of "neutral reducing steroids" in the urine is thought to bear a relation to the endogenous 11-oxygenated corticosteroid production. The reducing substances in the urine have hitherto sometimes been loosely termed "11-oxysteroids."

TABLE I
Electrolytes MEq per l. serum (Case 1)

Day		Na	K	Total base	CO ₂	Cl	Prot. gm. per cent	Ht	Urea mgm. per cent
15		125	3.5	135	30.4	86	5.4	38	51
19						87			
20	10 mgm. DOCA o.d.								
21	10 mgm. DOCA o.d.		2.7	138		90			
22	10 mgm. DOCA o.d.								
23	10 mgm. DOCA o.d.				32.5	86			
24	10 mgm. DOCA o.d.		1.9		33.0	87			32
25	10 mgm. DOCA o.d.								
26		129	2.1	138	32.1	89	5.2	34	35
29		134	2.7	147	32.5	91	5.9	37	31
33		133	2.7	145	30.0	93	6.4	38	30
40			4.1			96			
44			3.5	145		101			
91		140	5.0	148	28.6	108	6.9		
97								36	28

fallen as low as 1.9 MEq. per l. There was no definite electrocardiographic evidence of potassium deficiency, but there was an increase of muscle pain suggesting the onset of the potassium deficiency syndrome, and it was decided that the desoxycorticosterone should be stopped after a further 24 hours. On cessation of desoxycorticosterone administration the serum potassium was still as low as 2.1 MEq. per l., whereas there had

only been a slight rise in serum sodium to 129 MEq. per l. and in chloride to 89 MEq. per l. The behaviour of the blood pressure was unexpected in that a fall occurred from 165/120 to 135/88 during this period and subsequently rose again.

The patient was then maintained for ten days on sodium chloride without adjuvant therapy, during which there was slight improvement in the

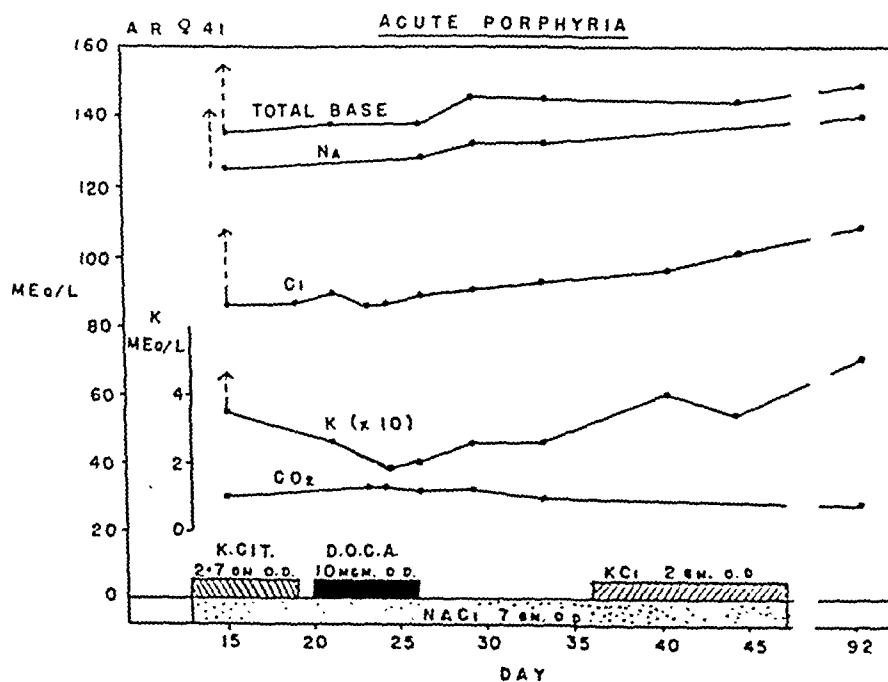


FIG. 1. THE EFFECT OF DESOXYCORTICOSTERONE AND OF POTASSIUM ON THE SERUM ELECTROLYTES (CASE 1)

EFFECT OF D. O. C. A. IN ACUTE PORPHYRIA.

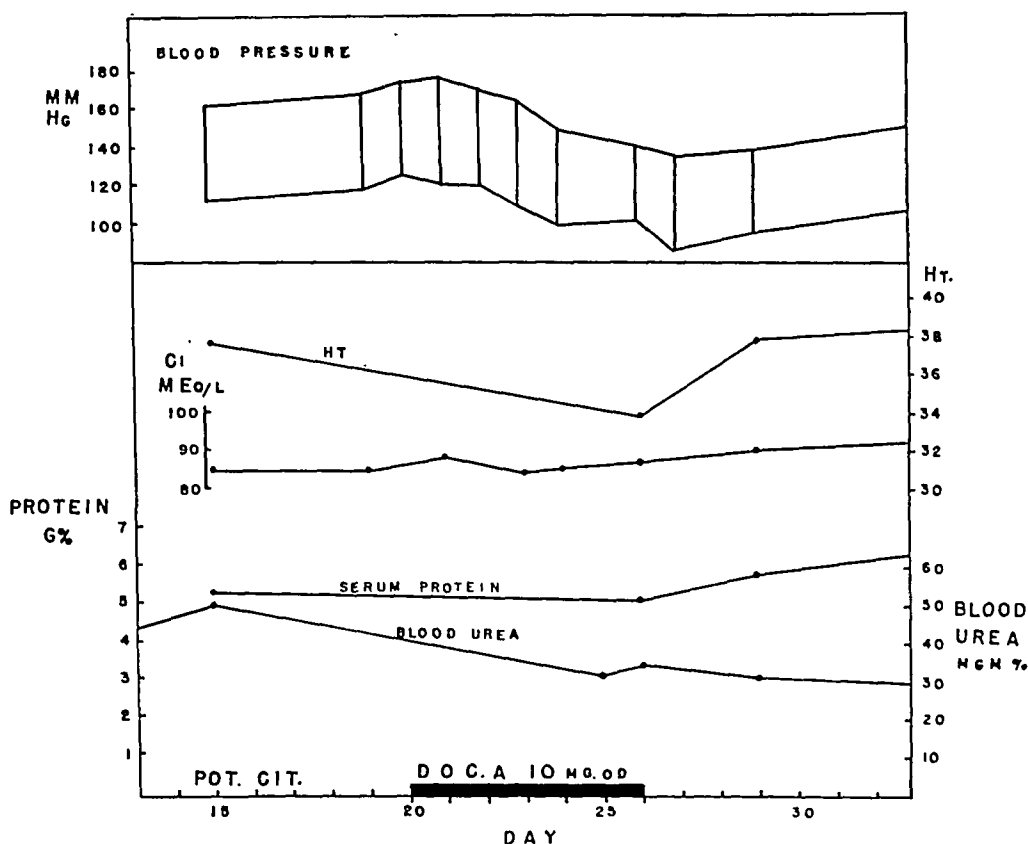


FIG. 2. THE EFFECT OF DESOXYCORTICOSTERONE ON SERUM CHLORIDE AND BLOOD PRESSURE (CASE 1)

sodium and chloride with a persistently low potassium. Potassium chloride was given in 2 gm. doses daily from the 36th day and thereafter there was a marked improvement with respect to potassium, chloride and sodium. This corresponded to a period of clinical improvement. The changes in the carbon dioxide combining power were minimal throughout these periods.

Excretion of urine chloride

It will be seen from Table II that in spite of moderate hypochloreaemia at the beginning of observation the urine chloride excretion amounted

to as much as 168 MEq. per day. It remained considerable throughout the period of study.

Excretion of "neutral reducing steroids" and response to adrenocorticotrophin

On the 18th and 21st days the excretions of urinary "neutral reducing steroids" were observed to be 0.62 and 0.54 mgm. per day, about twice the normal figure. At the 90th day this excretion had fallen to normal values and there was a small fall in 17-ketosteroid excretion which remained at low normal values (Figure 3).

On the 16th day a single dose of 24 mgm. adrenocorticotrophin² was given intramuscularly. This elicited after four hours a normal fall in circulating eosinophils of 91 per cent (Figure 3). The test was repeated on the 97th day and produced a smaller decrease, amounting to 60 per cent. It should be noted that in the first case the total fasting eosinophil count was only 59 per

TABLE II
Urine excretion of chloride (Case 1)

Day	DOCA	Average Cl	Urine volume
	10 mgm. per day	MEq per day	ml.
18-20	—	168	2593
21-23	+	248	3527
24-26	+	80	1742
27-29	—	166	2805
30-32	—	166	2687

² I am indebted to the Armour Laboratories, Chicago, for the adrenocorticotrophin.

c.mm., whereas in the second case it had risen to 236 per c.mm., thus behaving inversely to the excretion of "neutral reducing steroids." The data concerning uric acid metabolism show that in the initial test the uric acid excretion was high, the fasting uric acid/creatinine ratio being 0.71. In the second test the excretion had fallen to normal, the ratio being 0.46. On the first occasion the response in urinary excretion of uric acid to adrenocorticotrophin was small, but this was offset by a sharp rise of 17 per cent in the plasma uric acid level. These events occurred at a time when the blood urea was elevated to 51 mgm. per 100 ml. On the second occasion the response of urine uric acid excretion was on the low side of normal, the urine uric acid/creatinine ratio rising from 0.46 to 0.69 after adrenocorticotrophin. In this test the alteration in plasma uric acid was minimal.

The interpretation of these changes would appear to be as follows (Forsham *et al.* [12]).

1. In the early period there was increased adrenocortical activity suggested by:

(a) Increased excretion of "neutral reducing steroids" in the urine.

(b) High fasting urine uric acid/creatinine ratio.

(c) Low fasting eosinophil count.

2. In the early period there was adequate adrenal cortical reserve function, shown by:

(a) Normal fall in eosinophil count after adrenocorticotrophin.

(b) A sharp rise in the plasma uric acid level, associated with evidence of decreased renal function and absence of increase in urine uric acid excretion at a time when uric acid excretion was already enhanced.

3. In the later period, after complete subsidence

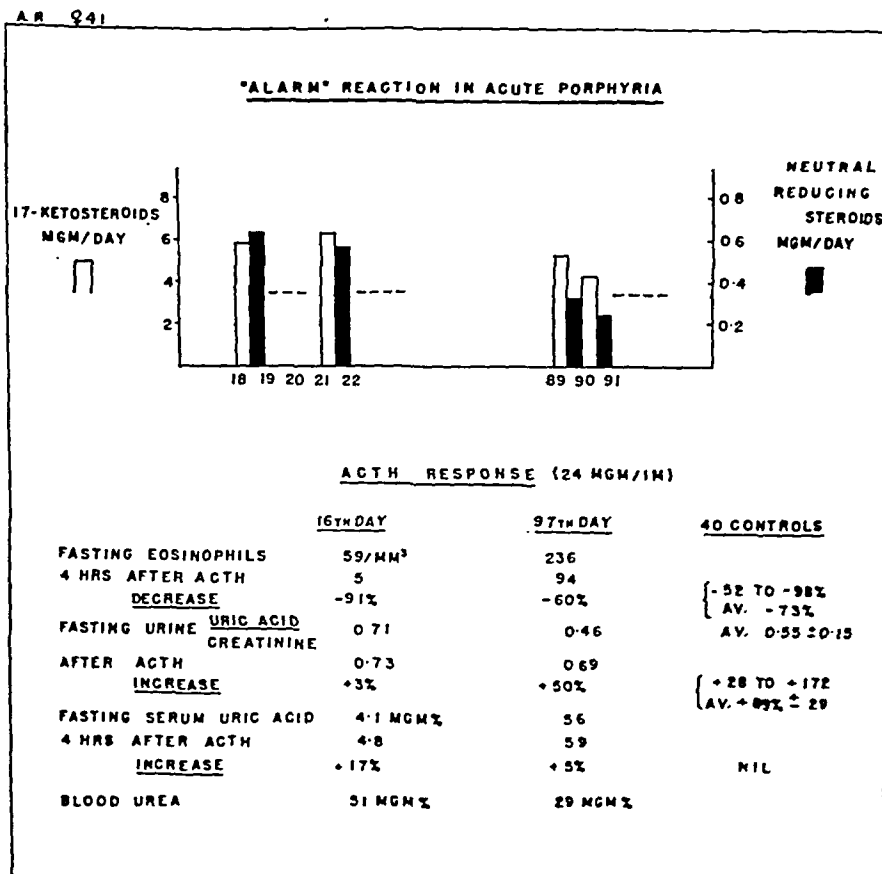


FIG. 3. "NEUTRAL REDUCING STEROID" EXCRETION AND RESPONSE TO ADRENOCORTICOTROPHIN (CASE 1)

FIG. 4. ADRENAL CORTEX (CASE 3) $\times 180$

of the attack of porphyria, the adrenal cortical activity had returned to normal, indicated by:

(a) The normal excretion of "neutral reducing steroids."

(b) The fall to normal of the fasting urine uric acid/creatinine ratio.

(c) The increase in the fasting eosinophil count.

4. In the later period there was still an adequate reserve in adrenal cortical function, shown by:

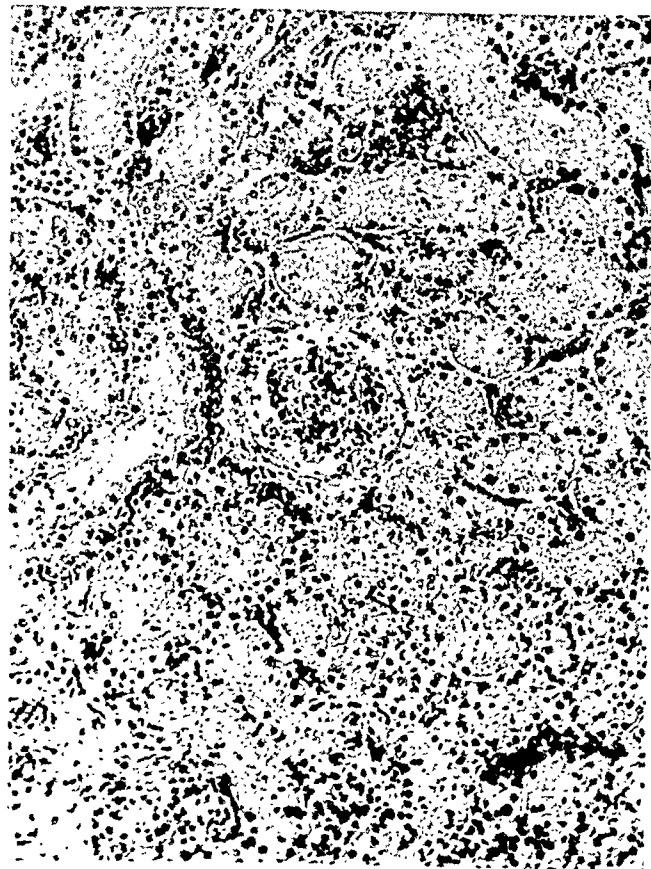
(a) Normal fall in eosinophil count after adrenocorticotrophin.

(b) Low normal response in the urine uric acid/creatinine ratio to adrenocorticotrophin.

Postmortem findings in the adrenals in Cases 2 and 3

Examination of the adrenals from two further cases of acute porphyria (Cases 2 and 3) gave evidence of cortical hypertrophy. In Case 2 the left adrenal weighed 7 gm. and in Case 3 the total weight of both adrenals was 16 gm. Histological examination of sections stained with haematoxylin and eosin showed hypertrophy of the cortical tissue, and the cortical widths in Cases 2 and 3 were approximately 2 and 4 mm., respectively. In addition a greater number of cortical cells, especially in the fasciculata, showed a well-stained uniform cytoplasm and absence of lipoid

vacuolation of the cytoplasm than is seen in the normal cortex (Figure 4). There is also evidence of increased capillary dilatation in these areas.

FIG. 5. KIDNEY, HAEMATOXYLIN AND EOSIN $\times 150$ (CASE 4)

Postmortem findings in the kidneys in cases of acute porphyria

Histological examination of the kidneys from Cases 2, 3 and 4 showed similar appearances. There was congestion of the capillaries and Cases 2 and 3 particularly showed some swelling of the capsular epithelium with the presence of eosinophilic material within the capsular space (Figure 5). The convoluted tubules and particularly the second convoluted tubules, showed severe cloudy swelling and atrophic changes in places. The lumina of some of the tubules contained epithelial debris. The nuclei showed varying degrees of pyknosis which in places was marked (Figure 6, Case 3).

Ultraviolet microscopy of the material from Case 2 showed a ring of red fluorescence in many of the tubules and a diffuse reddish fluorescence among necrotic epithelial elements contained within the tubules. In Case 4 there was a fairly intense red fluorescence in the convoluted tubule cells and in the debris within the tubules.

DISCUSSION

The responses of fall in eosinophils and of increased uric acid excretion to adrenocorticotrophin have been shown by Forsham *et al.* (12) to be due primarily to 11-oxy-steroid secretion by an adrenal cortex capable of stimulation. By these criteria Case 1 would appear to have an adequate adrenal cortical reserve, both during the height of the attack of porphyria and after its cessation. The increased excretion of "neutral reducing steroids" in the urine during the acute phase is suggestive of increased adrenal cortical activity and is supported by finding some elevation of the fasting blood sugar at the conclusion of the adrenocorticotrophin test. Such a response to the adrenal is typical of the "alarm reaction" of Selye (14) to non-specific somatic trauma, and may occur in the course of any acute episode in a disease process.

The question of a parallel increase of desoxycorticosterone-like activity on the part of the adrenal under such conditions is not settled, but it is probable that the part played by the adrenal in the "alarm reaction" is mediated through the anterior pituitary (Selye [14], Long [15]) and there is evidence suggesting that anterior pitui-



FIG. 6. KIDNEY, HAEMATOXYLIN AND EOSIN (CASE 3)

tary adrenocorticotrophin can under certain conditions stimulate increased desoxycorticosterone-like activity (Prunty *et al.* [3]). The response of Case 1 to desoxycorticosterone was investigated with the idea that if endogenous desoxycorticosterone deficiency existed a dramatic response should be obtained as observed by Loeb *et al.* (16) and by Thorn *et al.* (17) in Addison's disease. On the other hand, if endogenous production was adequate the response would be less dramatic. The observed response was poor especially when the potentiating effect of potassium withdrawal is also considered. The most marked effect appears to have been in decreasing an already low serum potassium, which latter in itself is unlikely in the presence of endogenous deficiency of desoxycorticosterone. In the case reported by Abrahams *et al.* (1) the serum potassium was also below, rather than above, normal. The observation of a fall in blood pressure during the administration of desoxycorticosterone is an unusual one, and not a typical response. The effect of desoxycorticosterone in increasing chloride excretion has so far only been observed in



FIG. 7. ADRENAL CORTEX FROM A CASE OF CUSHING'S SYNDROME, EXCRETING 8.2 MG. OF "NEUTRAL REDUCING STEROIDS" PER DIEM (FOR COMPARISON)

man under conditions where available desoxycorticosterone is excessive, either from excessive administration (Zierler and Lilienthal [18]) or in Cushing's syndrome (Soffer *et al.* [19]). It was also found that improvement in the state of serum electrolytes and of the patient's general condition continued and even became more marked when potassium chloride was subsequently given.

The postmortem state of the adrenals in Case 2 and Case 3 suggests increased adrenal cortical activity. Adrenal hypertrophy also appears to have been present in the case of Abrahams *et al.* (1). The adrenals of these cases may be compared with the adrenal cortex from a man with Cushing's syndrome excretion up to 8.2 mgm. per day of "neutral reducing steroids" (Figure 7). The findings in these glands, in the light of evidence of increased cortical activity in Case 1, are compatible with those of Rogers and Williams (20).

If the sodium and chloride deficiency observed is not due to adrenal failure, some other explana-

tion must be sought. Examination of the kidneys from Cases 2, 3 and 4 shows mild changes reminiscent of those seen in the so-called "renal anoxia" syndrome described by Macgrath, Harvard and Parsons (21), or perhaps better termed "lower nephron nephrosis" by Lucké (22). The renal changes have been well described by Darmady *et al.* (23). In this syndrome failure by the kidney to retain chloride has been found to occur by Humphrey and Jones (24), and even potassium deficiency can apparently occur (Bywaters and Stead [25]). It has been shown by Thorn, Koepf and Clinton (26) that sodium and chloride retention is not induced by desoxycorticosterone when certain types of renal lesion are present, and where marked depletion of these ions may be found (Peters, Wakeman and Lee [27]). A mild degree of non-protein nitrogen retention also frequently occurs in porphyria and was observed in Cases 1, 2 and 4. The possibility that the renal tubules in Case 1 may have been involved in pyelonephritic lesions must be also taken into account. Pyelonephritis was observed to occur in the case of Abrahams *et al.* (1). It is clear that further investigations of these points are required with more attention to the functional renal pathology.

SUMMARY

1. A phase of decreased plasma sodium and chloride has been studied in acute porphyria. This reacted poorly to administration of desoxycorticosterone acetate.

2. Data concerning the eosinophil counts, uric acid excretion and "neutral reducing steroids" excretion indicated increased adrenal activity during the acute phase of the disease. The response by the adrenal to adrenocorticotrophin stimulation showed an adequate adrenal cortical reserve function during the acute phase and in convalescence.

3. From examination of adrenals of cases of acute porphyria it is evident that cortical hypertrophy can occur and there is histological evidence that secretory activity may be increased.

4. In the light of present knowledge the correlation of the various phases of adrenal cortical activity are discussed and it is concluded that the disordered electrolyte metabolism is not primarily adrenal in origin. Abnormalities present in histological section of the kidneys suggest the

necessity for further investigation of renal function in this disease.

APPENDIX

Case notes

Case 1. A 42-year-old woman complained that for nine days she had suffered from anorexia and nausea. She was an orphan, and had two healthy children. Since an operation for prolapse eight years previously she had had attacks of cystitis. For a week there had been marked asthenia and several attacks of vomiting with small quantities of vomitus, and she complained of an aching pain in the lower abdomen. The attack coincided with one of her menstrual periods which were normal. She had noticed nocturia. The patient was a dark-haired individual with some generalised increased pigmentation and mild facial hirsuties. There was marked tenderness in both inguinal fossae, but no guarding, and constipation was severe. Blood pressure was 150/90, pulse rate 100 and temperature ranging up to 99° F. The urine contained a trace of albumin and a few pus cells of which 80 per cent were polymorphs. The blood urea was 44 mgm. per 100 ml. She was given potassium citrate on account of her urinary symptoms.

After seven days penicillin therapy was commenced but without effect. After 13 days of increased pain and developing rigidity in the lower abdomen exploratory laparotomy was carried out without significant findings. The patient had become mentally poorly co-operative and was slightly hallucinated.

Her urine then was shown to contain porphobilinogen and uroporphyrin and the diagnosis of acute porphyria was established, the uroporphyrin excretion ranging from 12 to 35 mgm. per day. (A study of the porphyrin excretion is being fully reported elsewhere [McSwiney, Nicholas and Prunty, 1949] [28].)

On the 15th day her blood pressure had risen to 160/115 and the pulse rate varied between 120 and 130 for the following three weeks. The plasma protein was 5.4 gm. per cent, albumin 3.3 gm. per cent, globulin plus fibrinogen 2.1 gm. per cent. Serum cholesterol was 222 mgm. per cent with 68 per cent ester; alkaline phosphatase 2.4 units, thymol turbidity 4 units; blood urea 51 mgm. per cent and icteric index 4 units. On the 16th day the urobilinogen excretion was 2.9 mgm. per day, and 10.4 mgm. per day on the 28th day. It fell to less than 1 mgm. per day from the 22nd day onwards. At the conclusion of the adrenocorticotrophin test on the 16th day and after 18 hours' fast, the blood sugar was 131 mgm. per cent, but glycosuria was never found. Values for serum electrolytes are recorded in Table I.

Potassium citrate was withdrawn on the 18th day and desoxycorticosterone acetate 5 mgm. b.d. given intramuscularly from the 20th to 25th days inclusive, and she was receiving 7 gm. of added sodium chloride. At the end of this period of six days she complained of increasing widespread muscle pain and a constrictive feeling in the chest. There was a generalised fine somatic tremor. The electrocardiogram was interpreted as being within

normal limits on two occasions, but reading was difficult on account of the tremor. Since the patient's condition had apparently deteriorated and the potassium level was so low (Table I) D.O.C.A. was withdrawn.

On the 29th day the plasma protein was 5.9 gm. per cent, albumin 3.0 gm. per cent; serum cholesterol 311 mgm. per cent, 64 per cent being ester; alkaline phosphatase 2 units; icteric index 5 units. There was a *B. coli* bacilluria which failed to improve with penicillin therapy.

On the 36th day she was given potassium chloride 2 gm. daily in addition to the sodium chloride. From this time she started to improve clinically and the pulse rate gradually returned to normal in the following five weeks. At the end of this period she had greatly improved, the blood pressure had fallen to 130/60, and urea clearance was 75 per cent of normal. The *B. coli* bacilluria was cleared up with mandelate therapy. After a further four weeks she was discharged well.

Case 2. A girl aged 16 was admitted to hospital with convulsions after a week's acute abdominal pain. Pulse rate was 110, temperature 100.5° and blood pressure 120/80. Blood urea was 43 mgm. per cent, plasma protein 7.5 gm. per cent, albumin 3.4 gm. per cent. Urine urobilinogen two plus. The urine contained porphobilinogen, and uroporphyrin amounting to 15 mgm. per day. The attack subsided in three weeks.

Six months later the patient returned with abdominal pain, soon after the onset of which menstruation commenced. After two weeks ascending paralysis appeared and the patient ultimately died of bronchopneumonia and intercostal paralysis. Investigations during the attack showed 26,000 white cells; blood urea 62 mgm. per cent, plasma protein 6.5 gm. per cent, albumin 3.2 gm. per cent. There was a slight increase of urine urobilinogen and an occasional granular cast. The urine uroporphyrin excretion ranged up to 68 mgm. per day.

The chief postmortem findings were areas of focal necrosis in the liver which was shown to contain uroporphyrin by ultraviolet microscopy and analysis. It also contained porphobilinogen. The renal and adrenal findings are described above. Detailed investigations of the case have already been reported. (Prunty [29].)

Case 3. A man aged 20 complained of marked asthenia for several weeks. Six months previously he had an attack of paralysis accompanied by a dark urine, and three months previously an attack of abdominal pain with similar urine.

During the present attack the pain was generalised with tenderness in the iliac fossae. There was slight conjunctival icterus. After the onset of mild paralytic symptoms he died suddenly in ten days. The serum sodium was 126 MEq. per l. and blood urea 34 mgm. per cent nine days before he died. Porphobilinogen and porphobilin were present in the specimen of urine examined and a moderate amount of uroporphyrin as the zinc metal complex.

At autopsy the myocardium was found to be thin, but otherwise normal. The liver showed areas suggestive of early focal necrosis. The hepatic cells contained numerous brownish pigment granules, particularly in the cen-

tral portion of the lobules. The adrenals and kidneys have been described above.

Case 4. A man aged 26 for two months noticed a darkening in the colour of his urine. At birth a meningocele was present. He was admitted to hospital with a diagnosis of intestinal obstruction for two days. Constipation was complete. He was in shock with a very low blood pressure, pulse 140, and temperature 101° F. The blood contained 18,500 white cells; N.P.N. 66 mgm. per cent, carbon dioxide combining power 41 MEq. per l., chloride 88 MEq. per l. The urine had a specific gravity of 1020, no albumin, but hyaline casts and unidentified brown crystals were present. There was a great deal of porphobilin present in the specimen of urine examined, with much porphyrin metal complex which could not be further identified with the amount of material available. There was a two plus reaction for urobilinogen.

The patient died at the end of a week and autopsy showed congenital absence of the left kidney with double renal pelvis and ureter on the right. There was extreme gut distention and much fluid present. The liver appeared normal. Histologically the liver showed the presence of numerous non-iron staining brownish granules in the liver cells. Ultraviolet microscopy revealed intense red fluorescence in these areas. The pigment appeared to be concentrated particularly in the cells in the central portions of the lobules, particularly about the central vein. The parenchyma in the centres of some of the lobules showed early degenerative changes. The kidney is commented upon in the text above.

I am indebted to Drs. Adams and Harewood Little for material from Cases 3 and 4.

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TREATMENT OF NEPHROSIS WITH CONCENTRATED HUMAN SERUM ALBUMIN. I. EFFECTS ON THE PROTEINS OF BODY FLUIDS¹

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The preparation of a pure, concentrated human serum albumin by Cohn and his associates (1) offers a unique opportunity for the study and treatment of patients with a deficiency of serum albumin. The homogeneity of this material permits the tracing of the administered protein (2), while the infrequency of untoward reactions on the patient and the freedom from risk of homologous serum jaundice allow the use of albumin in cases in which the physician would hesitate to administer whole plasma or plasma substitutes (3-5).

The severe deficiency of albumin in the nephrotic syndrome presents a strong indication for albumin therapy, but treatment with albumin has produced quite variable effects on hypoproteinemia and edema (4-9). The variability of clinical and chemical response in different patients and in the same patient at different times led us to examine in detail the effects of the administration of concentrated human serum albumin to patients with the nephrotic syndrome. The results call for a re-examination of some of the basic assumptions concerning the hypoproteinemia and edema of nephrosis.

PATIENTS

Thirteen patients with the characteristic features of the nephrotic syndrome have been treated. No patients with marked hypertension or renal insufficiency were included.

The patients were put to bed and given a diet calculated to contain protein, 1 gram/kg. and sodium 0.5 m.eq./kg. per day, with fluids ad lib. When the fasting weight

became stationary for three or more successive days, treatment with concentrated human serum albumin in 25% solution was begun. Albumin was administered through a 20 or 21 gauge needle, at a rate of 25 grams in 40 to 60 minutes.

Twenty courses of albumin therapy have been given, varying from three to six days in length. The usual dosage was 50 grams per day, but daily dosage was modified in four cases to meet individual problems. Only 25 grams per day were given to one older patient as a precaution against possible cardiovascular complications. Dosage was reduced to 25 grams per day for two women who complained of severe headache after larger doses. Seventy-five grams per day were given to one patient in whom 50 grams had failed to induce diuresis, but the only additional effect was an increased proteinuria.

The duration of treatment was guided by the effects. In the early cases, we received the impression that the maximal diuresis occurred in the first few days of treatment, so that if diuresis failed to appear in three to five days, treatment was stopped. If a good diuresis appeared, treatment was continued until the weight became stationary and/or edema disappeared. These principles were tentative and were determined to some extent by the availability of albumin at that particular time.

METHODS

These have been described in an earlier report (2). The following minor changes have been made. No attempt has been made to determine the plasma volume with T-1824 when fasting serum was visibly lipemic. Plasma protein concentration was determined by nitrogen content, which has also been used for albumin determination by sodium sulfate fractionation. Albumin concentration, measured by electrophoresis, cannot be calculated from the total protein concentration measured by nitrogen content, since the refractive index of lipid-rich plasma is higher than expected from its nitrogen content (10, 11). Since the electrophoretic analysis measures the proportion of colloid refractive increment contributed to serum by albumin, this percentage has been applied to the total colloid refractive increment of the serum to ascertain the refractive increment contributed by albumin. As the specific refractive increment of albumin is known, the albumin concentration can be calculated. This calculation yields a value for albumin concentration which is lower than the value obtained from the sodium sulfate fraction, which is known to be contaminated with globulin. The difference between the two values for albumin concentra-

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tion was constant in the same individual from time to time, but among eight different patients of this series the difference varied from 0.2 to 1.0 gram per 100 cc.

RESULTS

1. *Plasma volume.* Following each injection of concentrated albumin, there was a rapid expansion of plasma volume (Figure 1). The dilution of the blood was often clearly visible as lipemia and hemoglobin concentration declined. Measurement of the plasma volume with blue dye, T-1824, confirmed the hemodilution when the plasma was clear. If the plasma was even slightly lipemic, dilution increased light transmission very greatly, giving further evidence of the dilution of the blood but making the direct determination of dye impossible. The concentration of globulins in the plasma decreased after each injection of albumin (Figure 2). The concentrations of the globulins and of hemoglobin rose again in the intervals between injections.

The various methods agreed on the large magnitude of the increase of volume. The data obtained by several methods in one patient are presented in Figure 3. Volumes based on the fall

in hematocrit probably exaggerated the extent of the changes. Measurement of the hemoglobin concentration indicated a smaller change in plasma volume. The dilution of the blue dye T-1824 was also less than the dilution of the blood cell volume. Dilution of the plasma globulin (determined by electrophoresis) corresponded reasonably well with the other determinations. In calculations involving plasma volume, the dilution of T-1824 has been used.

Observation of the patient confirmed the increased blood volume. After each injection, the veins of the neck became fuller as the venous pressure rose a few centimeters of water. Transitory increases in pulse rate and blood pressure were common. A sensation of fullness in the head and neck was sometimes noted by the patient. Dyspnea sometimes followed the administration of albumin when the vital capacity was seriously reduced by pleural effusions and ascites.

When 50 to 75 grams of albumin were given daily, there was considerable individual variation in the duration of the effect on plasma volume (Figure 1). In some cases, the increase in volume was sustained for many hours. Subsequent in-

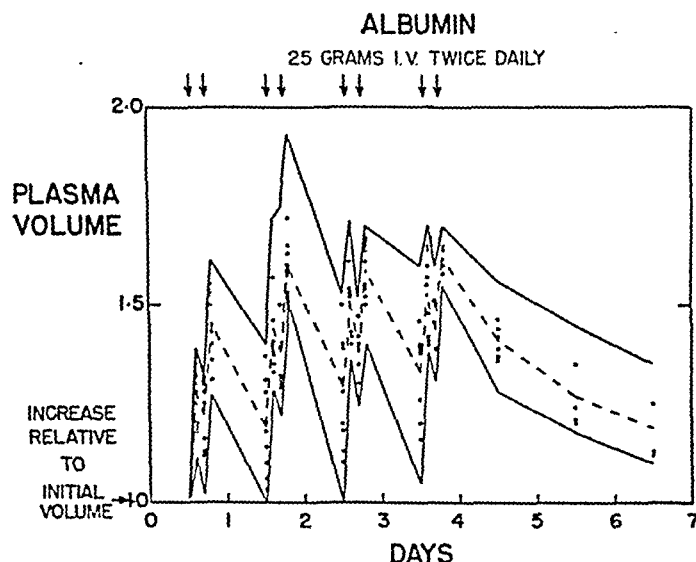


FIG. 1. EFFECT OF INTRAVENOUS ADMINISTRATION OF ALBUMIN ON THE PLASMA VOLUME

All volumes are related to the initial volume taken as unity. The heavy outlines show the range, and the broken line gives the average of measurements during 12 courses of treatment of nine patients. Note the large increase in plasma volume after each dose of 100 cc. of 25% solution of albumin.

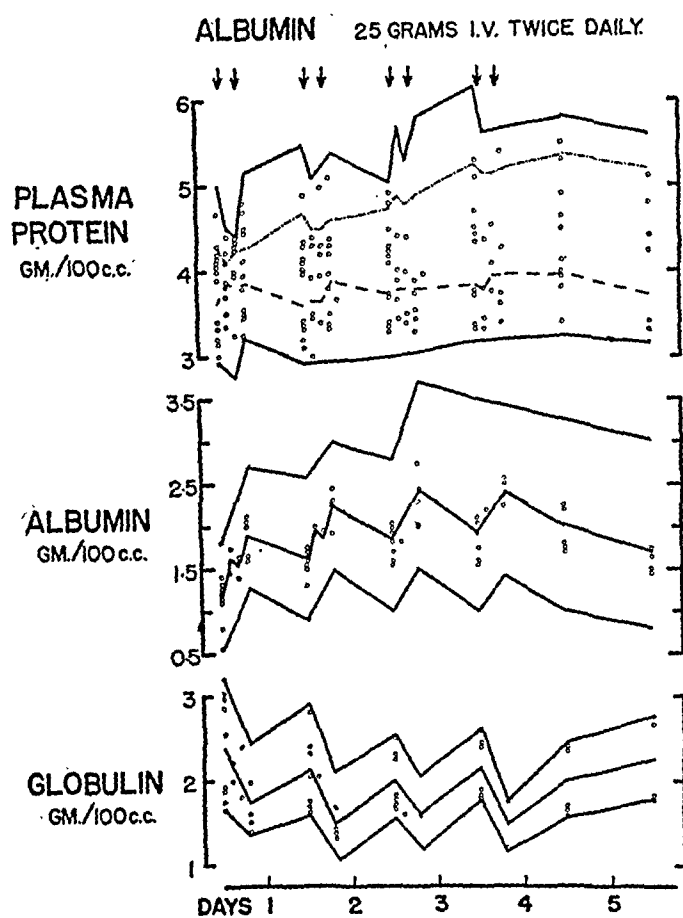


FIG. 2. EFFECTS OF INTRAVENOUS ADMINISTRATION OF ALBUMIN ON THE CONCENTRATIONS OF PLASMA PROTEIN, ALBUMIN, AND GLOBULINS

Data are from 14 courses of treatment of 11 patients. The heavy outlines give the range, and the middle lines the average of the individual cases. Each injection was followed by an increase in the plasma albumin concentration, while the globulin concentration fell. Treatment had little effect on the total plasma protein concentration in the ten instances whose average is shown by the broken line, but in four patients, there was a definite increase in the protein concentration, indicated by the dotted line. All of these four patients were free of edema at the end of treatment.

jections on the same day produced further increases, and the volume did not return to the base line overnight (Figure 4). In Case 1, a maximum was reached on the second day at a volume nearly double the control levels (Figure 5). In other cases, the maximum was reached more slowly. In a few instances, the plasma volume returned rapidly toward its usual level, and cumulative increases in volume on successive days were small or absent (Figure 1).

2. *Concentration of albumin and globulins in plasma.* In most of the patients studied, the concentration of albumin in the plasma was increased

most strikingly on the first two days of therapy (Table I). Subsequent injections produced much smaller changes in the concentration of plasma albumin, which fell quickly when therapy was stopped (Figure 2). A cumulative increase in albumin concentration beyond the first few days is possible when proteinuria is minimal and when diuresis follows (Table I).

The increased albumin concentration was reflected in a parallel rise in colloid osmotic activity of the plasma (Figure 5).

The change in the total protein concentration was less striking and frequently was negligible (Table I, Figure 2). When concentrated albumin solutions are injected intravenously, the added albumin draws into the plasma a large volume of extracellular fluid. The plasma globulins are diluted, and the concentrations of the various electrophoretic fractions fall in a uniform fashion as the albumin concentration rises. Since albumin has a higher osmotic activity than most of the proteins of nephrotic plasma, more water per gram of protein may enter the plasma than was originally present. Thus the total protein concentration may decrease after an injection of albumin, even though the osmotic activity of the plasma is increased by the rise in concentration of albumin (Figure 5). On later days of treatment, the total protein concentration tended to rise slowly, but there was consider-

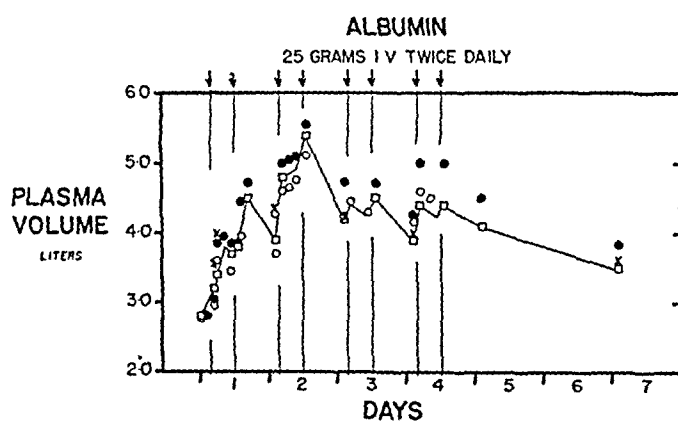


FIG. 3. CHANGES IN PLASMA VOLUME CALCULATED FROM DILUTION OF DYE, HEMATOCRIT, HEMOGLOBIN AND GLOBULIN, IN A PATIENT RECEIVING ALBUMIN (CASE 1)

The initial plasma volume was determined with the blue dye T-1824. Subsequent changes were calculated on the basis of dye (square), hematocrit (solid circle), total hemoglobin determined as cyanmethemoglobin (open circle), and globulin determined by electrophoresis (cross). All data agree on the large increases in volume. The hematocrit tends to exaggerate the change.

able variation from patient to patient. Two factors which influenced the changes in plasma protein and albumin concentration were variations in edema and the loss of albumin from the plasma.

3. *Effect of edema on the plasma protein concentration.* The lack of a sustained diuresis following treatment was apparently also a factor favoring dilution rather than concentration of the plasma proteins. The three patients whose plasma protein concentration approached normal were completely free of edema at the end of treatment (Figure 6). In general, the patients with the least edema showed larger increases in plasma protein concentration. When edema was massive, the changes in plasma protein concentration were smaller and less consistent in direction.

Diuresis can occur without an increase in plasma protein concentration, and with an increase in al-

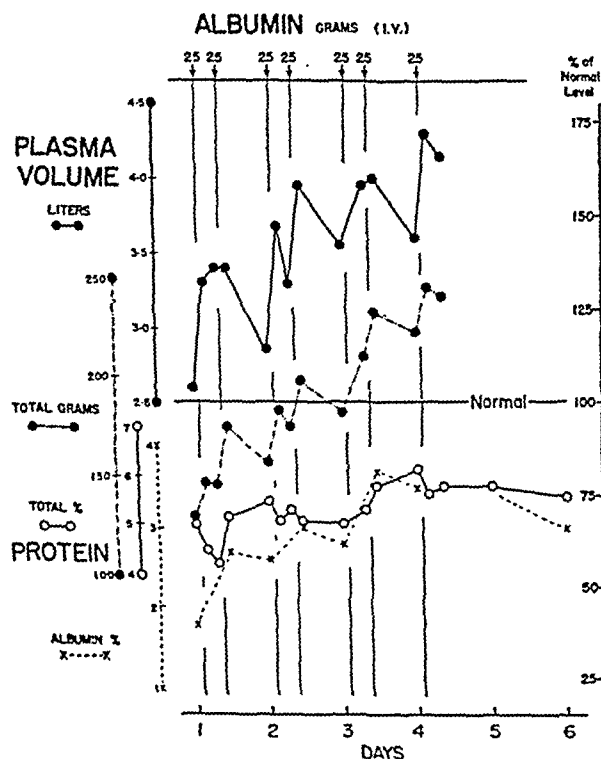


FIG. 4. EFFECTS OF TREATMENT WITH ALBUMIN ON THE PLASMA PROTEINS IN CASE 7

All data are plotted as per cent of normal.

The patient's total circulating protein was increased to normal levels after two days of treatment. Diuresis began on the first day and was complete by the end of the third day of treatment. On the third day, the plasma protein and albumin concentrations rose to the lower limits of normal.

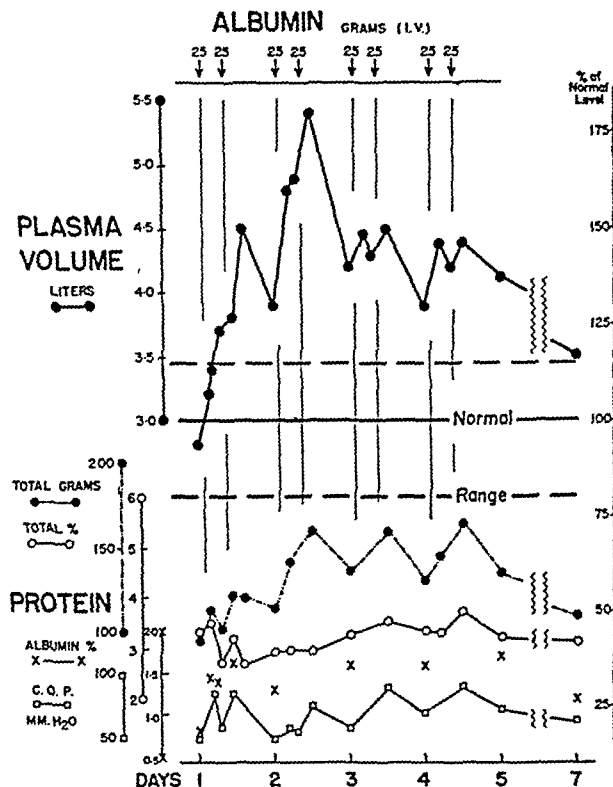


FIG. 5. EFFECTS OF TREATMENT WITH ALBUMIN ON THE PLASMA PROTEINS IN CASE 1

All data are plotted as per cent of normal.

The total circulating protein (solid circles, broken line) is the product of the plasma volume, and the plasma protein concentration (open circles, solid line). During treatment, the total circulating protein was increased almost to the normal range (equivalent to 6% protein and a normal plasma volume), but was so diluted by extracellular fluid that there was no appreciable increase in plasma protein concentration. The colloid osmotic pressure and albumin concentration of the plasma were approximately doubled, but the patient was still massively edematous at the end of treatment.

bumin concentration no larger than that observed in cases with persistent edema (Figures 6 and 7). In no instance, however, did the plasma protein or albumin concentration approach normal levels without a preceding complete relief of edema (Figure 6, Table I).

4. *Total circulating protein.* When the total circulating protein is calculated from the protein concentration and volume of the plasma, it is evident that the injection of concentrated albumin can effectively replace the deficit of circulating albumin. The total circulating protein was approximately doubled, reaching 75-125% of the normal

TABLE I
Summary of results of 20 courses of treatment with albumin

Case no.	No. 1		No. 2		No. 3		No. 4		No. 5		No. 6		No. 7		No. 8		No. 9		No. 10		No. 11					No. 12		No. 13	
	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	Std.	S-P	S-P	S-P	S-P	S-P	S-P	S-P	S-P		
Type of albumin†	4	3	3	4	5	5	4	4	3	3	4	4	4	4	6	6	6	6	4	4	5	4	3	4	5	5	3		
No. days of treatment																													
Dosage { Total grams Total grams/kg.†	50 150 200 2.7	50 150 200 2.0	50 150 200 2.7	50 200 3.6	50-75 300 5.5	25-50 225 4.1	50 200 3.3	50 200 3.3	50 150 2.4	50 300 5.4	50 200 3.2	50 300 4.4	25-50 175 4.4	25-50 175 4.4	50 300 5.4	50 300 5.4	50 300 5.4	50 300 5.4	25 100 1.4	25 100 1.4	50 250 4.8	25-50 125 2.4	50 150 2.9	25-50 150 2.9	50 250 5.2	50 150 3.1	50 150 3.1		
Plasma protein concentration { Initial, grams/100 cc. After 2 days End of treatment 2 weeks later	3.3 3.3 3.3 3.1	3.1 3.4 3.3 —	3.2 3.8 3.8 3.2	2.9 3.7 3.8 3.2	3.1 3.7 3.4 3.2	3.2 3.3 3.4 3.3	4.0 4.5 4.6 —	4.1 4.3 4.4 —	3.4 3.0 3.8 3.8*	4.7 4.2 5.0 5.0*	4.1 4.3 4.1 —	5.0 5.1 5.8 5.3*	5.0 5.1 5.8 5.3*	5.0 5.1 5.8 5.3*	4.2 4.2 4.4 4.4	4.2 4.2 4.4 4.4	4.5 4.2 4.4 4.3	4.5 4.2 4.4 4.3	4.1 — 4.9 —	4.1 — 4.9 —	4.3 4.8 5.3 5.1	4.2 4.1 4.5 4.3	4.3 — 4.3 4.2	3.9 — 4.1 —	3.3 3.9 3.8 3.6	3.3 3.2 3.3 —			
Plasma albumin concentration { Initial, grams/100 cc.† After 2 days End of treatment 2 weeks later	0.8 1.6 1.7 0.7	1.4 1.8 2.0 —	1.3 — 1.9 1.3	1.3 2.0 2.3 1.5	1.2 1.9 1.8 1.4	1.4 1.6 1.7 1.5	2.0 2.2 2.2 —	1.1 1.7 1.8 —	0.6 1.0 1.0 0.8*	1.2 2.0 2.2 —	1.1 1.7 1.8 —	1.8 2.8 3.5 2.7*	1.8 2.8 3.5 2.7*	1.8 2.8 3.5 2.7*	1.3 — 1.8 1.5*	1.3 — 1.8 1.5*	1.1 1.6 1.7 1.4	1.1 1.6 1.7 1.4	1.3 — 2.0 —	1.3 — 2.0 —	1.0 — 2.5 1.7	1.0 — 2.5 1.7	0.8 — 1.8 —	1.6 — 2.3 —	1.3 2.0 2.2 1.5	1.5 2.1 2.2 —			
Hematocrit { Initial, vols. % Minimum End of treatment	36 22 26	26 18 20	33 25 30	25 20 25	30 21 25	31 19 24	44 31 35	50 38 43	45 35 41	40 31 38	36 28 34	37 26 29	33 28 31*	33 28 31*	21 — —	25 24 29	46 31 41	46 31 41	39 41 —	39 41 —	29 21 31	32 27 29	29 23 —	37 29 —	48 38 51	27 27 —	38 25 28		
N.P.N. { Initial, mg./100 cc. End of treatment 2 weeks later	61 25 52	61 48 —	25 25 31	28 26 28	49 43 41	41 37 38	37 32 —	29 27 30	— — 38	21 — —	25 24 29	33 31 31*	33 31 31*	33 31 31*	21 — —	25 24 29	46 31 41	46 31 41	39 41 —	39 41 —	29 21 31	32 27 29	29 23 —	37 29 —	48 38 51	27 27 —	51 27 28		
Proteinuria { Control, grams/day Maximum End of treatment 2 weeks later	19 51 22*	14 33 —	17 40 16	12 40 16	13 65 13	13 49 14	5 10 —	9 39 9	20 58 18*	3 41 4*	6 — 6	4 11 7*	4 11 7*	4 11 7*	3 41 4*	6 — 6	5 26 7	5 26 7	13 23 —	13 23 —	4 22 4	4 11 5	5 19 —	9 32 —	18 70 16	8 28 —	8 28 —		
Body weight { Initial, kg. End of treatment 2 weeks later	92.7 91.4 93.2*	92.8 90.5 —	66.0 63.5 63.5	63.2 61.1 61.7	69.6 71.0 72.2	72.2 70.0 67.5	62.0 62.8 —	67.5 63.7 63.7	92.8 90.5 93.5*	68.5 61.5 60.0*	68.5 61.5 60.0*	41.3 39.8 40.3*	41.3 39.8 40.3*	41.3 39.8 40.3*	68.5 61.5 60.0*	68.5 61.5 60.0*	66.2 59.8 61.3	66.2 59.8 61.3	75.0 73.8 74.0	75.0 73.8 74.0	61.7 53.5 —	56.1 54.7 55.6	57.0 56.1 56.0	56.4 54.5 —	67.6 69.3 71.2	68.3 66.8 —	68.3 66.8 —		
Edema { Initial, grade** End of treatment	4+ 4+	4+ 4+	4+ 3+	4+ 3+	4+ 4+	4+ 4+	1+ 1+	3+ 0	4+ 4+	4+ 2+	4+ 2+	2+ 0	2+ 0	2+ 0	4+ 2+	4+ 2+	3+ 0	3+ 0	2+ 0	2+ 0	4+ 0	2+ 1+	3+ 2+	2+ 1+	4+ 4+	4+ 4+	4+ 4+		

* Follow-up period less than two weeks.

† "Standard" albumin contained sodium, approximately 400 m.eq. per liter. "Salt-poor" albumin contained sodium, approximately 80 m.eq. per liter.

‡ Dosage calculated in total grams/kg. edema-free body weight.

§ Concentrations of plasma protein, albumin and nonprotein nitrogen describe blood drawn under basal conditions on the morning after treatment. The level of albumin in blood drawn at the end of an injection may be as much as 50% higher than the figure given in this table (see Figure 2).

|| Albumin concentrations in italics are calculated from electrophoretic analyses, which yield results averaging 0.7 gram/100 cc. lower than the albumin concentration determined by the salting-out procedure used in the other measurements.

¶ Hematocrits at beginning and end of treatment are drawn under basal conditions, but minimum hematocrit invariably follows an injection of albumin.

** Edema is graded from 0 to 4 plus. Edema just detectable is graded 1 plus, definite and more extensive 2 plus, heavy and generalized 3 plus, massive 4 plus.

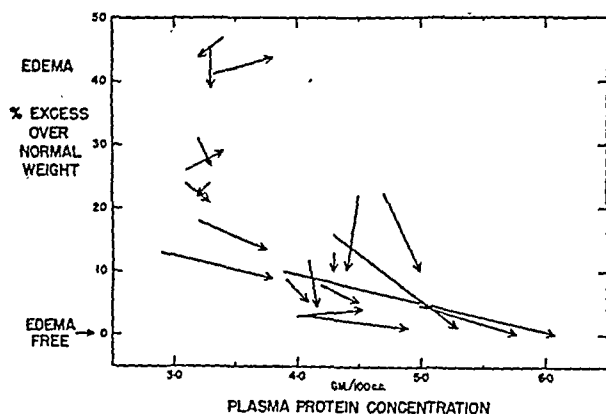


FIG. 6. RELATION BETWEEN CHANGES IN EDEMA AND IN PLASMA PROTEIN CONCENTRATION OF PATIENTS TREATED WITH ALBUMIN

Edema is expressed as per cent of usual body weight. Each arrow indicates the change in plasma protein concentration and in edema during a course of treatment with albumin, the head of the arrow representing conditions at the end of treatment. Note that patients with heavy edema generally had small increases or decreases in the plasma protein concentration after albumin treatment. A plasma protein concentration approaching normal was seen only when edema was eliminated.

total³ after two or three days of treatment (Figures 4 and 5). The proportion of albumin was usually increased until it equaled or exceeded the amount of globulin (Figure 2). This almost complete replacement of the plasma proteins was not regularly followed by a corresponding increase in plasma protein concentration because of the great dilution of the plasma.

5. *Duration of effects of treatment.* The effects of intravenous albumin on the circulating proteins persisted for only a short time after treatment was stopped. The plasma volume and albumin concentration declined as albumin was lost in the urine, usually reaching the control level within a week or two, depending on the rate of urinary loss. In chronic, stationary cases of nephrosis, the proportion of the blood proteins was often constant for months. When such patients were treated with albumin, the electrophoretic pattern returned with great accuracy to the pre-treatment picture. Persistent changes in the plasma proteins after treatment probably result from a more fundamental alteration in the pathological state.

³ "Normal circulating protein" = (plasma volume expected from height, edema-free weight, and sex) \times (7.5 grams of protein per 100 cc.).

In five such cases followed from four months to 2½ years, the proportion of albumin in the electrophoretic pattern varied less than 5% between any two analyses on the same patient. All five analyses of the serum of one patient and the two analyses of serum of another patient showed a proportion of albumin within $\pm 1\%$ of the mean. The proportion of albumin, reproducible within the error of the method, was grossly increased during treatment with albumin, but returned within four to 30 days (average 14 days) to the pre-treatment level.

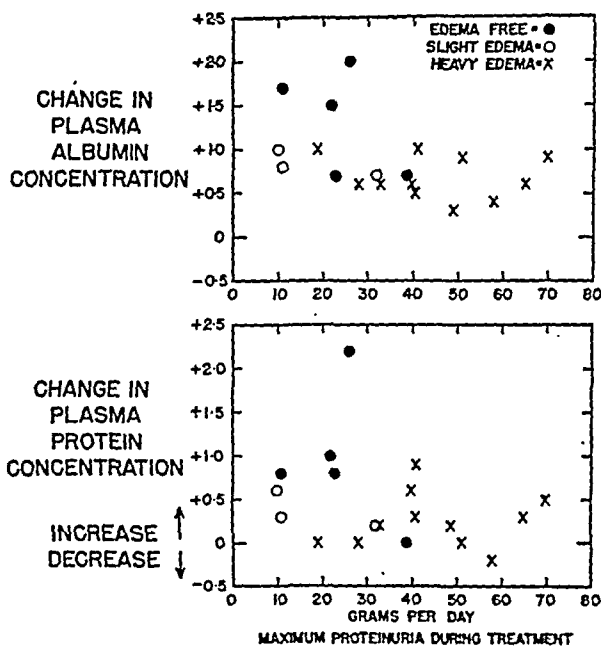


FIG. 7. THE RELATION BETWEEN PROTEINURIA AND CHANGES IN PLASMA ALBUMIN AND TOTAL PROTEIN CONCENTRATIONS

Each point represents a course of albumin treatment, and indicates the change in concentration of plasma albumin or protein during treatment, compared with the amount of protein lost in the urine on the later days of treatment when proteinuria was maximal. Essentially the same relationship is noted when the per cent of total injected protein lost in the urine during treatment is charted in place of maximal proteinuria. The degree of edema at the end of treatment is indicated by the symbols as heavy (X), slight (open circle), or absent (solid circle). Note the relatively small effect of the large variation of proteinuria on the change in plasma protein concentration. Relief of edema was infrequent in patients who developed massive proteinuria, and was quite variable in patients with little proteinuria. The largest increases of plasma albumin and protein concentration occurred in patients who eliminated their edema while they retained protein.

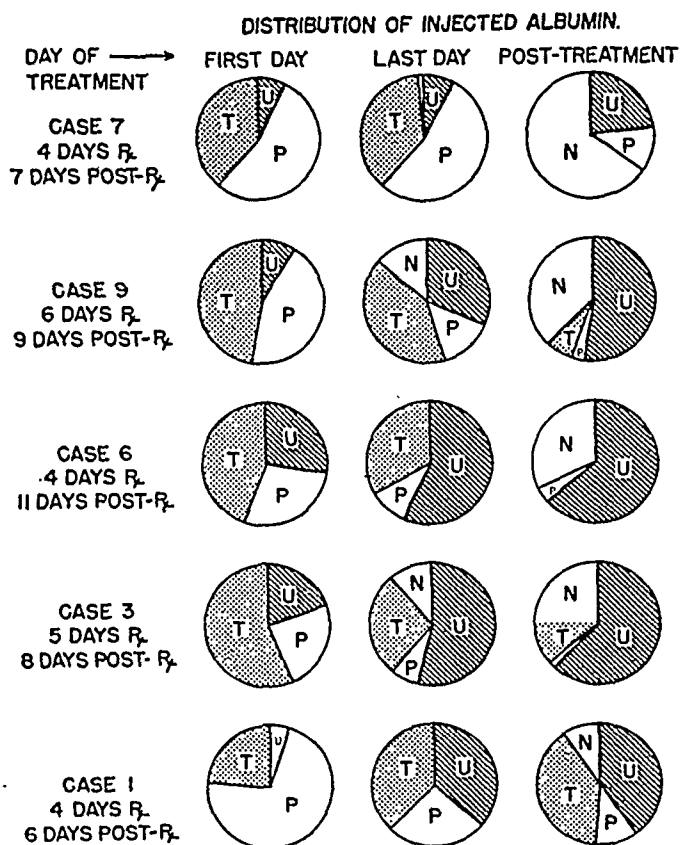


FIG. 8. FATE OF INJECTED ALBUMIN AFTER ONE DAY OF TREATMENT, AT THE END OF SEVERAL DAYS OF TREATMENT, AND SIX TO 11 DAYS AFTER THE END OF TREATMENT

P = Albumin retained in plasma in excess of control
 U = Albumin in urine in excess of control
 T = Administered albumin not found in plasma or urine
 N = Non-protein nitrogen from catabolism of albumin

The area of each circle represents the total dose of albumin administered, up to and including stated day. The distribution of the albumin is indicated by the areas of the various sectors.

Cases are arranged in order of response to therapy, from complete diuresis and normal protein level at the top of the chart (Cases 7 and 9) to failure to affect hypoproteinemia and edema (Cases 1 and 3) at the bottom of the chart. Note the relatively constant proportion of albumin stored in the tissues at the end of treatment. There is some correlation between amounts of albumin held in the plasma or lost in the urine, compared with the results of treatment in the first four cases. The failure of treatment in Case 1, however, can not be explained on this basis.

6. *Fate of injected albumin.* Much of the albumin given by vein disappeared from the circulation. A small part of the lost albumin appeared in the urine on the first day of treatment (Figure 8). If treatment was continued, a larger proportion was lost in the urine, reaching a plateau on the third or fourth day (Figure 9). In more

severe cases, the entire daily dose of albumin was lost in the urine after the first few days of treatment, but in milder cases, most of the administered albumin was retained in the body (Table I).

Between 33% and 55% of the injected albumin could not be found in the plasma or in the urine at the end of treatment (Figure 8). This protein must have passed into the extracellular fluid, and a part may have entered the cells, where it could be stored or burned. A few efforts were made to locate the lost protein. Samples of effusions and fluid before and after treatment were obtained when edema was not relieved by treatment. Analysis of these fluids for protein indicated that the injection of albumin increased the protein content of effusions and of interstitial fluid, but that both initial and final concentrations were very low. In Case 12, edema fluid, collected from each leg by Southey's tubes, contained 0.055% and 0.049% protein before albumin was given. The administration of 25 grams of albumin increased the concentration to 0.064% and 0.056% in the two legs. The protein concentration fell to the control level within four hours in the right leg, from which fluid continued to drain at a rate above 1 liter per day. The flow of fluid from the left leg fell to less than half the control rate for 48 hours, while the protein concentration remained at a level of 0.063%, and then the flow increased again, with a corresponding fall in protein concentration to the control level. This sort of data suggests that in the tissues, as well as in the plasma, shifts of fluid may affect the concentration of protein. One may recall a similar effect in Drinker's studies on the protein content of lymph. The important point is that the injection of albumin appeared to make only an infinitesimal change in a minute concentration.

The effect on the protein concentration of ascitic fluid was also very small. In Case 8, 150 grams of albumin in three days raised the protein concentration of 4 liters of ascitic fluid from 0.1% to 0.3%, while the weight dropped 1 kg., the plasma protein concentration fell slightly, and the plasma albumin concentration rose by approximately 65% above its initial level. In Case 12, 250 grams of albumin in five days raised the protein concentration of ascitic fluid from 0.1% to 0.2%. Although edema increased steadily despite treatment in this patient (see Table I), only 1 liter of ascitic

fluid could be obtained at the end of treatment; doubtless, more was present, but not a great collection.

Rough calculation from these data indicates that the amount of protein lost into the extracellular fluid would hardly account for all of the lost protein. This conclusion might also be inferred from the similar loss of albumin into the tissues, whether the patient is massively edematous or virtually edema-free (Figure 8). It seems likely that some of the protein was stored intracellularly. Some attempts were made to study such storage by the method of comparing nitrogen, potassium, and phosphorus balances, used by Albright, Reifenstein, and Forbes (12). The results were confused by the major changes in excretion of these elements, due to alterations in renal function and diuresis which followed albumin therapy. These changes will be commented upon in connection with nitrogen balance. At the end of two weeks,

most of the injected albumin nitrogen could usually be accounted for as excess albumin and non-protein nitrogen in the urine (Figure 8).

7. Effect on nitrogen balance. Study of the total nitrogen balance fails to describe adequately the changes during albumin therapy of nephrosis. It is important to separate the protein and non-protein fractions, which show different and sometimes opposite changes. It is also necessary to take into account changes in the volume and composition of body fluids which follow the administration of albumin (Figure 10).

Most of the albumin administered on the first day of treatment is retained in the body, but it has already been shown that more and more of the injected albumin may be lost on later days of treatment (Figures 8 and 9). When treatment is stopped, protein is lost in the urine in amounts considerably above the control level for several days. The excess protein in the urine is almost

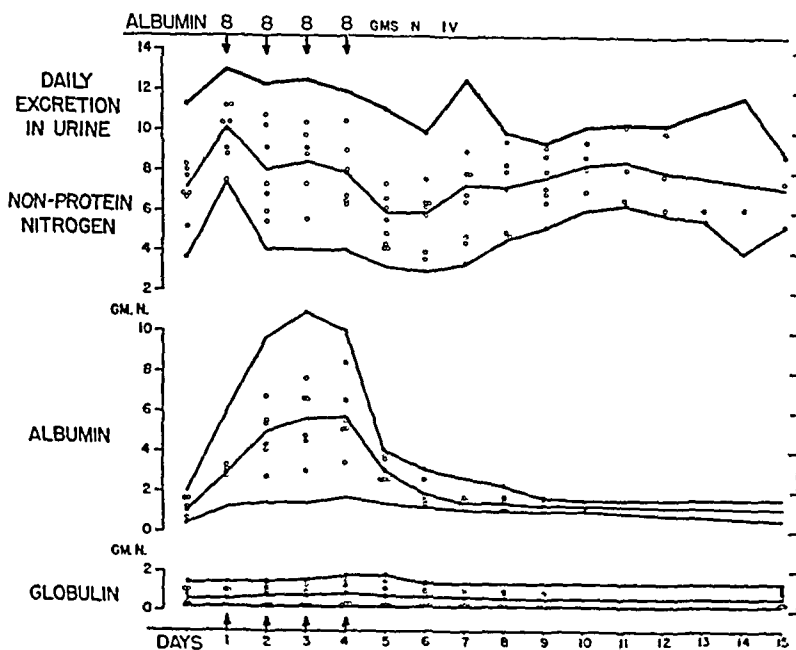


FIG. 9. EFFECTS OF ALBUMIN ADMINISTRATION ON THE EXCRETION OF NON-PROTEIN NITROGEN, ALBUMIN, AND GLOBULIN IN THE URINE

The outer lines show the range, and the central line indicates the average for nine patients.

Note the immediate increase in urinary non-protein nitrogen on the first day of treatment, and the fall below the base-line after the end of treatment. The increased protein in the urine, rising to a plateau after several days of treatment, is composed almost entirely of albumin. The increased nitrogen excretion during the second week is probably related to the catabolism of albumin stored during treatment.

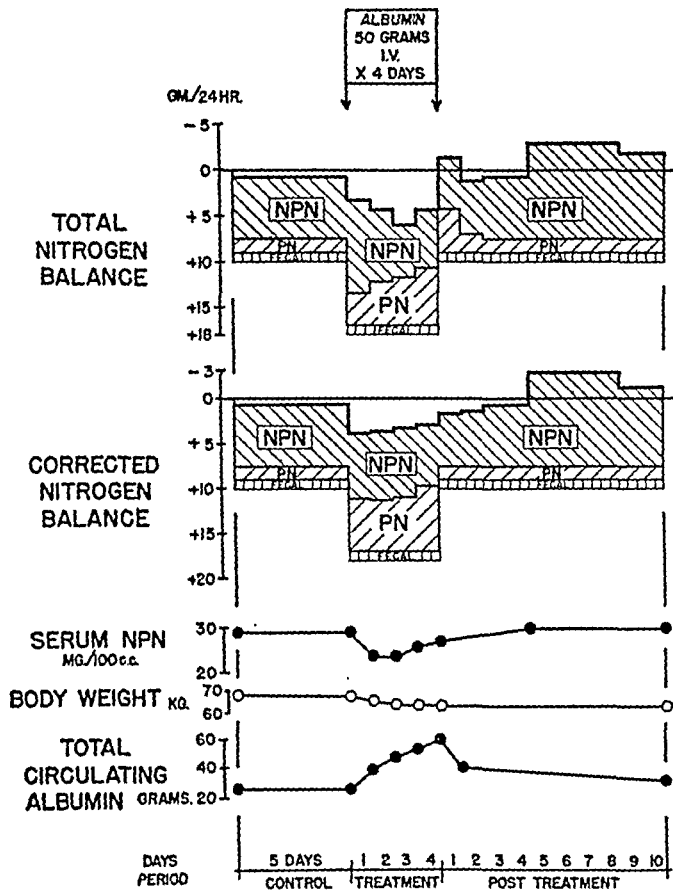


FIG. 10. EFFECTS OF ALBUMIN THERAPY ON NITROGEN BALANCE

In both balance charts, intake is plotted down from the base line (0) to the bottom of the shaded area. The nitrogen excreted by several routes is indicated as fecal, protein nitrogen (PN), and non-protein nitrogen (NPN). If the sum of these excretions falls below the base-line, the patient is in positive balance, i.e., intake exceeds output. If the total rises above the base line, the patient is in negative balance.

The upper chart represents total nitrogen balance, which reflects not only the metabolism of protein, but also changes in the volume and composition of the body fluids. In the second chart, the total balance is corrected for changes in body water, non-protein nitrogen concentration, and total circulating albumin, which are plotted below on an equivalent scale in terms of the grams of nitrogen which they represent. In this correction, non-protein nitrogen which is removed from the body fluids by diuresis and improved renal function is subtracted from the nitrogen excretion, since it does not represent any change in protein catabolism, but simply a shift from body fluids to urine. The nitrogen which accumulates in the body fluids after treatment is added to the nitrogen excretion, where it would appear if renal function were more efficient. Similarly, albumin which accumulates in the plasma during treatment, only to be excreted in the urine after treatment, is calculated as if it had not been retained. In the corrected chart, the storage and delayed catabolism of albumin in the cells can be seen, free from the extracellular and renal changes which complicate the total balance chart.

entirely albumin, as globulin excretion is little affected.

The changes in excretion of non-protein nitrogen (NPN) are different in several respects (Figures 9 and 10). At the beginning of albumin therapy, there is an increased excretion of NPN. The NPN concentration of the blood usually falls, averaging 7 mg. per 100 cc. lower at the end of treatment, with the largest decreases from blood levels above 30 mg. per 100 cc. After the end of treatment, the excretion of NPN falls temporarily below the control level, and the NPN concentration of the blood increases.

The initial rise of non-protein nitrogen in the urine can usually be related to the excretion of body water and to a fall in the NPN concentration of the body fluids and does not represent an increased catabolism of protein⁴ (Figure 10). The exact reverse of this effect may occur when treatment is stopped. Within two weeks after albumin therapy, an excess of nitrogen equivalent to the injected albumin is usually excreted or can be accounted for in the increasing volume and NPN concentration of the body fluids. These changes give an irregular appearance to the level of NPN excretion (Figures 9 and 10) which can be resolved into three phases: (a) increased renal excretion, (b) decreased renal excretion, (c) increase due to albumin catabolism. If the crude nitrogen balance is corrected so as to eliminate changes in volume and concentration of NPN and albumin in the body fluids, it is possible to visualize the storage and delayed catabolism of the albumin administered. Figure 10 shows the relative magnitude of these changes in a patient with good renal function and a moderate diuresis.

8. *Effects of loss of albumin in the urine.* The loss of albumin in the urine during treatment varied considerably from patient to patient. In some cases, the greatest daily loss was less than 20% of the injected albumin, but in other cases, most or all of the albumin was wasted in the urine. One might assume that this variability in loss would be reflected in the increase of plasma protein and albumin concentration during treatment. It is evident from the data in Figure 7 that variations in

⁴ This artificial change in external nitrogen balance appears to be caused by the increased glomerular filtration rate and diuresis which usually occur on the first days of albumin therapy (2, 18).

proteinuria have little systematic effect on the increase in plasma protein concentration, and only a slightly more dependable effect on the increase in albumin concentration during albumin replacement. This poor correlation probably is simply another indication that dilution of the added protein suppresses any large increase in concentration unless diuresis occurs. When proteinuria is relatively small and when diuresis occurs during treatment, a much larger increase in albumin and/or plasma protein concentration may follow (Figure 7).

The difference between the curves of total circulating protein in Figures 4 and 5 is probably the best indication of the effect of variation in proteinuria. In Figure 5, increasingly heavy proteinuria blocked further increase in total circulating protein after two days of treatment. In Figure 4, minimal loss from the plasma allowed cumulative increases in the total circulating protein on each day of treatment. Heavy proteinuria can thus greatly reduce the efficiency of albumin replacement and presumably may reduce the probability of a diuresis (Figure 7).

DISCUSSION

Concentrated human serum albumin has proved to be a useful therapeutic agent in five of our 13 cases of the nephrotic syndrome treated for brief periods. The relief of edema, with or without elevation of the plasma protein concentration, was followed by a considerable physical and psychological improvement. The effects of albumin on the blood proteins were temporary, but the reaccumulation of edema could be prevented or delayed by restriction of sodium intake. No fundamental change in the course of the disease has been apparent after albumin therapy. In these and many other respects, our results confirm the studies of Janeway (4, 5) and Thorn (6).

Some of the experimental observations during treatment with albumin support certain current views of the pathologic physiology of the nephrotic syndrome. For example, the effect of increased colloid osmotic activity of the plasma on fluid exchange at the capillaries was clearly demonstrated by the increased plasma volume after an injection of concentrated albumin.

Heavy loss of protein in the urine was obviously a serious drain on the patient's circulating pro-

tein. The loss of protein in the urine might not appear serious in the control periods, but when albumin was given intravenously, the increased plasma albumin concentration was reflected in an increased proteinuria. In general, heavy proteinuria during treatment occurred in patients who showed a very low plasma albumin concentration and/or a heavy proteinuria before treatment, indicating a high degree of renal permeability to albumin. In such patients, the loss of over half of the injected albumin in the urine greatly reduced the efficiency of plasma albumin replacement. In other patients with little albumin in the urine and a higher plasma albumin concentration before treatment, effective replacement of plasma albumin could be achieved quite economically (Table I).

The large variation from patient to patient in urinary loss of protein seemed to have a surprisingly small effect on the effects of treatment. Patients with massive proteinuria showed increases in plasma protein concentration nearly as large as those shown by patients who lost only a small amount of the injected protein in the urine. The other routes of loss from the plasma ("tissues") absorbed a fairly constant proportion of the injected protein. The variable fraction of albumin remaining in the plasma was apparently diluted to approximately the same concentration in nearly all cases. When diuresis occurred, however, *concentration* of the added protein could be achieved, and it was in this group of patients who lost their edema that the highest plasma protein concentrations were reached. These patients generally retained much of the injected albumin, but some other patients failed to eliminate their edema even though they retained equally as much of the injected albumin.

These findings do not support the concept that the edema of nephrosis is the simple consequence of a deficiency in albumin. It is necessary to distinguish clearly between replacement of the albumin deficit in grams of protein, and increasing the plasma protein concentration in grams per 100 cc. The albumin deficit can usually be made up by intravenous administration of albumin. To increase the concentration of plasma albumin and protein to normal is a much more difficult matter if diuresis does not occur.

In the presence of an excess of extracellular fluid, it seems difficult to raise the plasma protein concentration, since fluid dilutes the added albumin to a concentration approximating that of the original plasma. The administration of albumin thus increases the volume rather than the protein concentration of plasma. There are two obvious methods of increasing the plasma protein concentration under these circumstances. One solution is to remove the excess of extracellular fluid so as to permit the increase in osmotic pressure without excessive dilution of the blood, but this is not a simple matter if the kidneys fail to excrete the fluid. The other possible way to increase protein concentration is an apparent absurdity, the substitution of an osmotically inactive protein. Albumin may be osmotically ineffectual if its concentration increases equally in the plasma and in the interstitial fluid. This situation occurs in the ascites of some patients with hepatic cirrhosis (7, 13, 14) and in the edema fluid of patients with an unusual form of idiopathic hypoproteinemia (7). What little evidence we have indicates that the protein concentration of the edema fluid in nephrosis is too low to influence appreciably the effective colloid osmotic pressure of the plasma proteins. The logical conclusion of this line of thought is that injected albumin can be concentrated in the plasma of an edematous patient with nephrosis only if an effective diuresis ensues. If a diuresis does not follow, an anomalous situation occurs, in which the total circulating protein approaches normal, but the protein concentration remains very low, while the plasma volume becomes abnormally large. This mobilization of fluid at the peripheral capillaries without a corresponding diuresis throws the responsibility for the intractable edema directly on the kidney's failure to eliminate the excess of fluid.

The conjunction of plasma albumin depletion and of the retention of fluid is sufficiently common in nephrosis to justify the usual explanation of edema on the basis of hypoproteinemia. The observant clinician, however, will note that major changes in edema may occur without comparable changes in plasma protein or albumin concentration (15-17). We now have evidence suggesting that such changes in edema are due to variations in renal excretion of sodium and water, which are not explicable as a sequence of proteinuria, depletion of plasma albumin, lowered plasma colloid

osmotic pressure, and escape of fluid from the capillaries (18, 19). Change in the volume of the plasma and interstitial fluids must be considered as an independent factor which is equally as important as change in total circulating protein in determining the plasma protein concentration.

The importance of sodium retention in the production of edema is emphasized by the greater diuretic effect of salt-poor albumin (5, 6), suggested by the data in Table I. The sodium balance of these patients will be the subject of another report. It may suffice to say here that many patients with nephrosis have great difficulty in excreting sodium in certain phases of their disease and that treatment with albumin may or may not have a large effect on the excretion of sodium. In an occasional patient, sodium restriction seems to be unimportant (see Case 2, Table I) during a brief course of treatment with albumin or during a spontaneous relief of edema. In most patients, however, it appears to be necessary to limit the intake of sodium if albumin is to be used most effectively as a diuretic.

The questions of optimal dosage and duration of treatment with albumin were not examined in this study. Two relevant pieces of information may be noted, however. The very large increase in plasma volume, the increased venous pressure, and the dyspnea occurring in patients with vital capacity already reduced by effusions, all suggest that doses larger than 50-75 grams per day should be given with the greatest caution and abandoned at the first signs of cardiac or respiratory difficulty. The loss of efficiency of albumin replacement after the first two to four days of treatment suggests that if some improvement fails to occur within a few days, treatment with albumin is likely to prove a very slow, costly and inefficient diuretic. It would seem easier and safer to remove the fluid with Southey's tubes if intensive or prolonged treatment with albumin appears likely to be risky or inefficient. The most important function of albumin is to replace the deficit of plasma proteins. If this replacement does not result in diuresis, more efficient methods of disposing of the edema may be indicated.

SUMMARY

1. The administration of concentrated human serum albumin in dosage of 50 grams per day for

three to six days to patients with the nephrotic syndrome regularly results in a large increase of plasma volume and of total circulating protein.

2. The proportion of albumin in the plasma is considerably increased.

3. The plasma protein concentration is often not significantly increased, even though the total circulating protein is replaced to nearly normal levels, because of the great dilution of the blood by fluid mobilized from the tissues and not excreted in the urine. This result was observed in six of the 13 patients.

4. Five patients were essentially free of edema after four to six days' treatment. These patients showed a larger average increase in concentration of plasma protein and albumin than patients with persistent edema showed, but there was a large individual variation.

5. We have not observed a plasma protein or albumin concentration approaching normal levels without a preceding complete diuresis. Elimination of the bulk of the protein-free edema fluid is apparently necessary before the serum protein concentration can be raised to normal levels.

6. Diuresis can occur without an increase in plasma protein or albumin concentration greater than that observed with persistent edema. Small increases in the colloid osmotic pressure evidently suffice to mobilize a large amount of edema, *provided that the kidneys excrete the mobilized fluid.*

7. The replacement of plasma albumin is hindered to a variable extent by the loss of albumin in the urine, which increases as the plasma albumin concentration is increased by successive days of treatment. When proteinuria is massive, the efficiency of protein replacement becomes very poor after the second or third day of treatment. We were unable to increase the plasma protein concentration to a useful degree in any case with massive proteinuria. When proteinuria is slight, cumulative increase in total circulating proteins can be maintained for a longer period, and the likelihood of diuresis appears greater.

8. The increase in plasma protein and albumin concentration after treatment with albumin is only slightly larger in patients with smaller amounts of proteinuria, since the protein retained in the plasma is diluted to nearly the same extent as the protein originally present. When edema is eliminated, the

concentration of proteins and albumin in the plasma may increase more strikingly.

9. At the end of treatment, a fairly constant proportion of the administered albumin cannot be found in the urine or plasma. Some of this albumin appears in effusions and edema fluid, when present, but another portion is probably stored in the cells of the body. At the end of two weeks, all of the nitrogen injected as albumin can be accounted for as excess albumin and non-protein nitrogen in the urine.

CONCLUSIONS

1. Concentrated human serum albumin is an effective therapeutic agent for the temporary replacement of the deficit of circulating albumin in the nephrotic syndrome.

2. The efficiency of replacement is obviously reduced in patients with massive proteinuria.

3. It seems very difficult to increase the plasma protein concentration in the presence of an excess of edema fluid of low protein content because of the great dilution of added protein.

4. It is suggested that removal of edema may be a necessary step in increasing the plasma protein concentration, as well as vice versa.

5. Both the edema and hypoproteinemia of the nephrotic syndrome are aggravated by the failure of the kidney to excrete excess fluid, and are therefore not necessarily corrected by replacement of plasma albumin deficit.

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MUCOLYTIC ENZYME SYSTEMS. VIII. INHIBITION OF HYALURONIDASE BY HUMAN BLOOD SERUM DURING THE NORMAL MENSTRUAL CYCLE AND PREGNANCY¹

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While evidence has been presented in preceding publications of this series that the hyaluronidase inhibiting property of blood serum is increased in infectious diseases of both bacterial and viral origin (1-4) and in cancer (5), changes that accompany certain normal physiological states have not been investigated.

It is well known that hyaluronidase plays an important role in fertilization by enabling the sperm to disperse the follicle cells surrounding the ovum (6-8), and that normal serum can inhibit this effect (9). Another aspect of particular interest in the light of the finding of Wattenberg and Glick (10) that certain of the steroid hormones inhibit the activity of hyaluronidase *in vitro*, would be alterations in the serum inhibitory level accompanying hormonal changes. Dorfman (11) has stated that men of reproductive age have a significantly lower range of hyaluronidase inhibitor levels than a comparable group of women.

Since hyaluronidase plays an important role in the invasion of the animal host by various toxic and infectious agents (12), the possibility that the serum inhibitor has a protective function should be kept in mind. Rosahn *et al.*, (13) reported that pregnant rabbits were more resistant to vaccinia infection than the non-pregnant, and Aycock (14) claimed that castrated monkeys were less susceptible to poliomyelitis infection after the administration of estrogenic hormone. Sprunt, McDearman, and Raper (15-16) demonstrated that pseudopregnancy or the administration of estrogenic substances resulted in inhibition of the spreading of india ink in the rabbit skin.

From the foregoing it would appear that the serum inhibitor might have an effect on fertilization, and the female sex hormones might possibly

influence resistance to infection through their affect on the hyaluronidase inhibitor. In order to gain some information relative to these points, as well as to observe changes with respect to certain normal physiological states, the present investigation was concerned with the variations that occur in the level of the hyaluronidase inhibitor in the blood sera of women during the menstrual cycle and pregnancy.

EXPERIMENTAL

Materials and Methods. The details of the preparation of the materials, the viscosimetric assay method used, and the calculation of results followed the recently described modifications (10) of the original procedure and mode of expressing inhibition (1). The hyaluronidase was prepared from bovine testicle and the hyaluronic acid from human umbilical cord. Hyaluronidase inhibition was expressed as the per cent inhibition effected by 0.02 ml. of blood serum under the conditions employed. The per cent inhibition was defined as $\frac{100(R - R_0)}{R}$ where (R_0)

is the time required to reduce the viscosity of the reaction mixture to one-half its original value, and (R) is the time required to reduce the initial viscosity to one-half after incubation of the 0.5 ml. of enzyme solution with 0.02 ml. of serum and 1.48 ml. of water for 10 minutes at 37.5° C. Because the inhibitor loses activity rapidly on standing at room temperature, it is important that the collection of samples be as uniform as possible. The blood was collected in clean tubes, allowed to clot, centrifuged, and the serum removed and immediately frozen. To eliminate differences in various batches of enzyme and substrate, all serum samples were stored at -20° C. until the collections were complete for a given experiment. All of the samples were then assayed the same day using the same enzyme and substrate preparations.

RESULTS

For the menstrual study, 10 healthy, regularly menstruating, unmarried women ranging in age from 22-28 were chosen as subjects. Since other diseases can influence the inhibitor values, any subject who developed a "cold," "gastrointestinal" upset, or any other disability was dropped from the study. The results of experiments on eight

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² Junior Research Fellow, National Institute of Health.

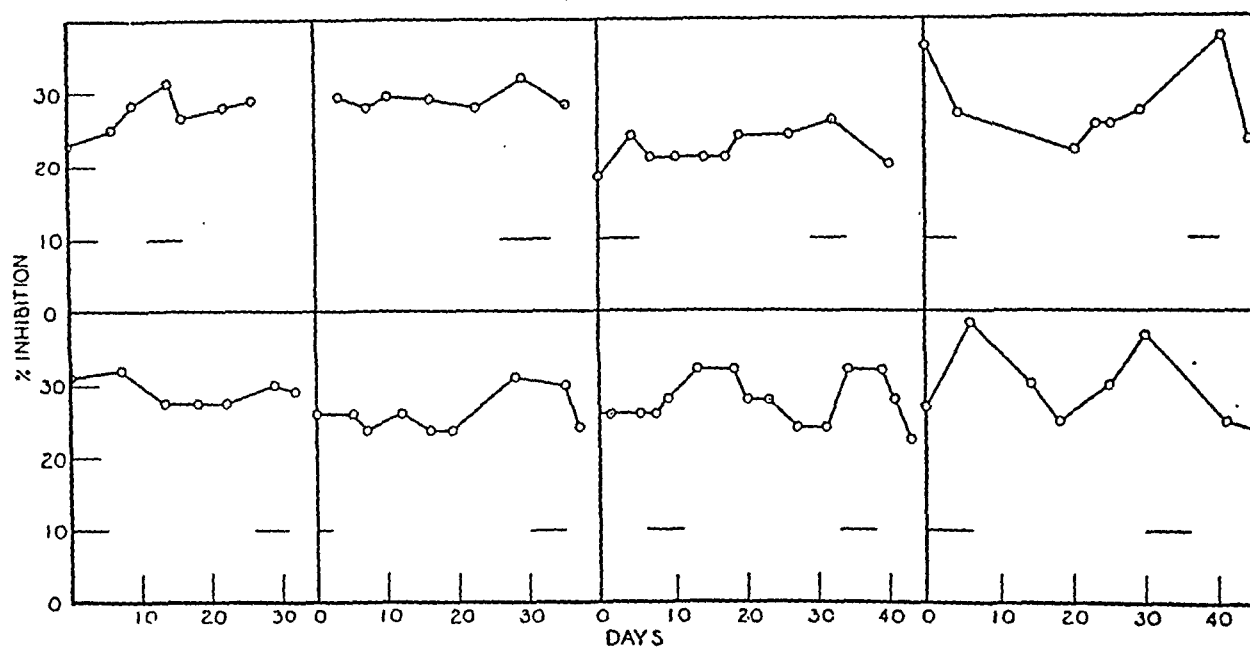


FIG. 1. PER CENT INHIBITION OF HYALURONIDASE BY HUMAN BLOOD SERUM DURING THE MENSTRUAL CYCLE

Horizontal lines indicate menstrual periods.

individuals who completed the study are shown in Figure 1. The highest values are found during or immediately after actual menstruation although the magnitude of the changes was subject to great individual variation.

In the pregnancy study the blood samples were

not collected from the same individual over the entire period of gestation. However, sera from groups of 10 women from each trimester of pregnancy were assayed and it was found that, although the inhibitor values increased somewhat during the latter period of gestation, the differ-

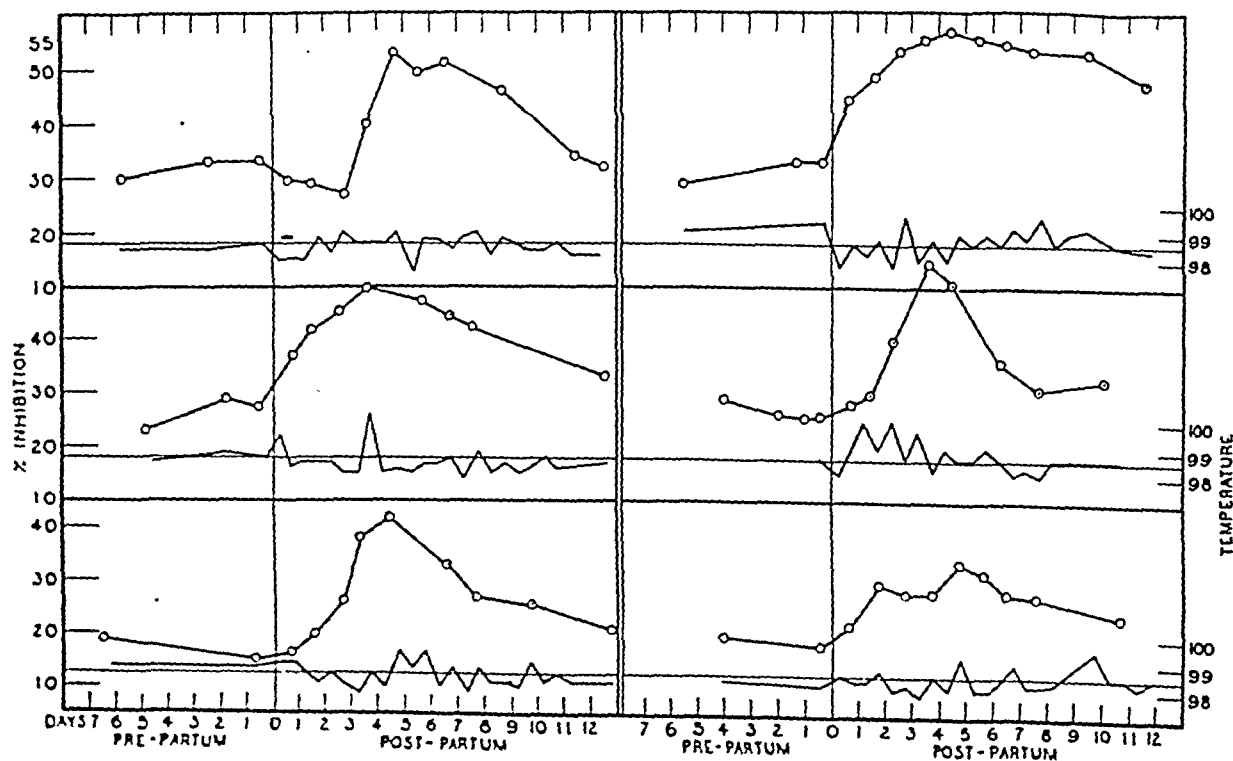


FIG. 2. PER CENT INHIBITION OF HYALURONIDASE BY HUMAN BLOOD SERUM DURING PRE- AND POST-PARTUM PERIODS, AND ACCOMPANYING ORAL TEMPERATURES

ences were not statistically significant. Since the more dramatic changes in the inhibitor level were found to take place just before and during delivery, studies on 16 uncomplicated pregnancies were carried out beginning about a week before the expected date of confinement and continuing for about 10 days post-partum. The data of six representative cases are shown in Figure 2. It is clear from the curves that there is a consistent and significant rise in the per cent inhibition, which reaches a peak in from two to five days post-partum and is almost back to the pre-partum level by the 10th day. The rise can be detected in some cases as early as two hours after delivery. The temperature curves, which contain the highest recorded temperature for each 12-hour period, show that this elevation is not consistently paralleled by a rise in body temperature.

DISCUSSION

Although no *abrupt* change in the level of the hyaluronidase inhibitor was observed during, or soon after, the expected times of ovulation, in some of the cases the lowest values did occur during the middle of the cycle. However, the significance of this change is in doubt since its magnitude was negligible in some of the women. Further doubt of a correlation between inhibitor levels and sex hormonal changes follows from a consideration of the fact that no appreciable inhibitor changes were observed during the course of pregnancy. The point deserving major emphasis is the unequivocal post-partum rise in the level of the serum inhibitor. The significance of this observation is not clear at present. There may be a common factor, such as tissue destruction, in the variations of inhibitor noted in this investigation and the increase reported for infection and cancer. This possibility is being pursued further.

SUMMARY

Studies were carried out on the inhibitory effect of blood sera from 10 normal women during various phases of the menstrual cycle, and from 16 women with uncomplicated pregnancies before, during, and after delivery.

It was found that the highest inhibitor levels tended to occur during menstruation, although the individual variation in this elevation was great. The data do not permit of a correlation with sex hormonal changes.

No significant change was found during pregnancy or labor. A marked post-partum rise was observed which reached its maximum from the second to fifth days after delivery.

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STUDIES ON AMINO ACID EXCRETION IN MAN. III. AMINO ACID LEVELS IN PLASMA AND URINE OF NORMAL MEN FED DIETS OF VARYING PROTEIN CONTENT¹

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Current emphasis on the use of proteins in the treatment of various disorders of the alimentary tract has stimulated a comparative study of certain aspects of protein metabolism in normal persons and in patients with gastro-intestinal disease. In addition to the usual nitrogen balances, microbiological assays were made of eight amino acids in food, acid-hydrolyzed whole plasma, tungstic acid filtrates of plasma (free amino acids), acid-hydrolyzed tungstic acid filtrates of plasma (free plus combined amino acids), hydrolyzed urine, and in feces. The amino acids measured were methionine, lysine, arginine, histidine, leucine, isoleucine, valine and threonine. The present paper deals with individual amino acid levels in the food, plasma, and urine of two normal adult men fed isocaloric diets of differing protein content. The data indicate that the fasting levels of amino acids in plasma are relatively constant, and that the excretion of amino acids in the urine does not fluctuate significantly despite considerable variation in amino acid intake.

METHOD OF STUDY

A. Subjects

Two healthy young men were studied in the metabolism section of Billings Hospital. In addition to the standard laboratory procedures, normal results were obtained for the following: (a) urea clearance test of renal function (1); (b) oral glucose tolerance; (c) hepatic function (2-9); (d) carbon dioxide ([10] p. 283), pH ([10] p. 796) and chloride ([10] p. 835), calcium (11), and phosphorus (12) content of serum; (e) plasma proteins (13); and (f) gastric secretion (14) (0.1 mg. histamine diphosphate per 10 kg. body weight).

Subject 1, J. D.; initial weight was 70 kg., height 175 cm. The basal metabolic rate, measured by the Benedict-Roth apparatus and calculated according to the standards of Boothby and Berkson, was +4; the basal calories were estimated as 1880.

Subject 2, R. G. H.; initial weight was 84 kg., height 187 cm. The basal metabolic rate was 0; basal calories were estimated as 1990. The blood and plasma volumes were measured by the Gibson-Evans method (15) at frequent intervals in this subject; the values were relatively constant, averaging 6198 and 2720 cc., respectively.

B. Diet

Normal activity was permitted. Fluids were allowed *ad libitum*, but the fluid intake and urine output were measured daily. The only medication consisted of a liquid vitamin B preparation given daily. Though the quantity of protein was varied, the caloric intake was kept uniform by adjustment of the carbohydrate and fat content of the diet. The number of calories was determined on the basis of the basal metabolic rate of each subject, with additional allowances for his usual activity. In Subject 1, the intake of calories was 3200, the carbohydrate ranging in the various periods from 403 to 477 gms. and the fat from 110 to 154 gms. In Subject 2, the intake of calories was 3190, the carbohydrate ranging from 415 to 475 gms. and the fat from 103 to 131 gms. Two to 4.0 gms. of table salt were added to the food each day. Crystalline methionine was added in periods 9 and 10; in all other periods the quantity of individual amino acids was varied by changes either in the amount or type of protein. Following a transitional interval of three days, each regimen was studied for two consecutive six-day periods.

C. Preparation and Analysis of Food, Plasma and Urine

The food was prepared in the kitchen of the metabolism section by dietitians trained in the problems of metabolic research. The food was weighed on a torsion balance before cooking; salt was the only seasoning; meats were broiled. The quality and quantity of the food were kept uniform throughout each regimen.

For analysis, a 30 to 40 per cent aliquot of a one-day supply of food (excepting pure carbohydrate and fat) was homogenized with distilled water in a Waring blender. A second aliquot of the same diet was prepared and analyzed approximately one week later; the results of both measurements were averaged.

At the end of each six-day period, following a 10-hour overnight fast, 100 ml. of venous blood were drawn, transferred to a screw-capped bottle containing 15 to 30 mg. of heparin, and mixed thoroughly. The blood was centrifuged and the plasma removed immediately. One-

¹ This study was supported in part by grants from the Evaporated Milk Association and American Dairy Association.

ml. samples were taken for the measurement of total nitrogen and for the assay of amino acids in acid-hydrolyzed whole plasma. Forty-ml. samples were used for the preparation of tungstic acid filtrates, as described by Hier and Bergeim (16). Aliquots of the tungstic acid filtrates were hydrolyzed with hydrochloric acid and assayed for total non-precipitable amino acids.

The urine was voided directly into glass containers and stored under toluene at ice box temperature (3-5° C.). The total six-day collection was combined and aliquots were taken for various analyses. Urea was measured by the urease method (17), free ammonia by the procedure described in Peters and Van Slyke (10) and the pH of the urine by the glass electrode method.

The total nitrogen of the food, plasma and urine was determined by a semi-micro-Kjeldahl technique, utilizing the Pregl-Parnas-Wagner apparatus and the digestion mixture of Campbell and Hanna (13). Amino acid assays were carried out by the microbiological method, as described elsewhere (18, 19). Recovery of measured amounts of pure amino acids added to the urine before acid hydrolysis ranged from 90 to 110 per cent. The validity of the microbiological procedures used in these studies has been confirmed by Toennies and Gallant (20). Leucine, isoleucine, arginine, histidine, valine and threonine were measured with *Streptococcus fecalis*; methionine and lysine with *Leuconostoc mesenteroides* P-60 as the assay organism.

RESULTS

The body weights of both subjects did not change significantly throughout the study. The total plasma proteins and the albumin-globulin ratios remained normal. The data for amino acids are presented in Tables IA and B. A change in concentration was considered significant if it exceeded the standard deviation² by two or more times.

Individual Amino Acids in Whole Plasma

The fluctuations in the methionine content of whole plasma in both subjects paralleled the varying intakes of this amino acid in food. For the seven other amino acids, there was no direct correlation between the quantities ingested and the plasma levels. The addition of crystalline DL-methionine to a diet adequate in this amino acid

² Standard deviation derived from expression:

$$S.D. = \frac{[M.D.]^2}{n-1}$$

S.D. = standard deviation; M.D. = mean deviation; n = number of values measured.

TABLE I-A

Intake in food, excretion in urine and fasting normal plasma levels of individual amino acids in two normal men

Periods	Subject 1 (J.D.)					Subject 2 (E.G.H.)				
	Intake in Food (gm./6 days)	Excretion in Urine (gm./6 days)	Fasting Amino Acid Levels in Plasma			Intake in Food (gm./6 days)	Excretion in Urine (gm./6 days)	Fasting Amino Acid Levels in Plasma		
			Whole Plasma (mg./dl.)	Free Amino Acid (γ/ml.)	Total Non-Protein Amino Acid (γ/ml.)			Whole Plasma (mg./dl.)	Free Amino Acid (γ/ml.)	Total Non-Protein Amino Acid (γ/ml.)
METHIONINE										
1,2	10.20	0.05	0.59	3.9	3.4	12.78	0.06	0.89	6.3	5.4
3,4	6.00	0.05	0.59	3.0	5.5	6.78	0.06	0.54	6.2	5.9
5,6	9.24	0.04	0.76	5.0	5.2	12.66	0.08	0.58	5.7	5.0
7,8	7.14	0.04	0.46	3.5	3.2	10.50	0.07	0.49	4.5	4.9
9,10	13.14	0.07	0.92	6.9	6.7	16.50	0.12	0.77	6.1	7.7
LYSINE										
1,2	27.84	0.29	7.9	23.5	45.0	35.58	0.85	8.4	28.2	65.0
3,4	9.90	0.32	6.2	24.2	45.0	16.56	0.73	8.66	21.7	56.5
5,6	28.98	0.36	7.7	27.0	47.0	32.60	0.95	8.17	27.9	69.3
7,8	18.72	0.31	6.4	20.8	36.1	28.80	0.83	8.40	24.7	60.2
9,10	18.72	0.37	6.98	28.0	60.5	28.80	0.80	8.17	31.2	69.0
ARGININE										
1,2	22.20	0.12	3.72	14.9	45.0	29.82	0.22	4.52	18.6	49.8
3,4	11.64	0.09	2.99	12.0	41.0	14.94	0.19	4.64	15.9	38.9
5,6	20.52	0.14	3.65	15.2	28.3	28.80	0.18	4.11	15.7	45.8
7,8	19.08	0.12	3.29	17.2	22.2	28.02	0.19	3.77	16.5	37.2
9,10	19.08	0.16	3.72	18.8	30.9	28.02	0.17	3.49	15.9	46.9
HISTIDINE										
1,2	11.52	0.77	2.23	15.9	24.7	16.14	2.76	2.12	13.2	14.5
3,4	6.18	0.44	2.20	17.7	21.6	7.68	1.64	2.20	13.5	22.7
5,6	11.52	0.80	2.34	12.5	21.6	15.90	2.36	2.26	14.4	27.5
7,8	10.80	0.72	2.14	17.0	20.8	13.08	2.44	2.20	14.2	25.7
9,10	10.80	0.90	2.40	17.5	20.5	13.08	2.48	2.27	17.0	25.8

- 6.0 gms. crystalline DL-methionine added in periods 9 and 10, supplementing diet of periods 7 and 8.
- All values are averages of data for two successive six-day periods.
- The apparently lower values for total non-precipitable methionine as compared with free amino acid values are due to the experimental error inherent in the microbiological method.
- The term "Total non-protein amino acids" should read "Total non-precipitable amino acids."

TABLE I-B

Intake in food, excretion in urine and fasting normal plasma levels of individual amino acids in two normal men

Periods	Subject 1 (J.D.)					Subject 2 (R.G.H.)				
	Intake in Food (gm./6 days)	Excretion in Urine	Fasting Amino Acid Levels in Plasma			Intake in Food (gm./6 days)	Excretion in Urine	Fasting Amino Acid Levels in Plasma		
			Whole Plasma mg./ml.	Free Amino Acid (γ /ml.)	Total Non-Protein Amino Acid (γ /ml.)			Whole Plasma mg./ml.	Free Amino Acid (γ /ml.)	Total Non-Protein Amino Acid (γ /ml.)
LEUCINE										
1,2	36.24	0.12	6.58	24.0	37.0	41.82	0.19	6.81	27.8	50.8
3,4	21.72	0.10	5.99	27.9	35.0	27.72	0.20	7.50	26.3	59.8
5,6	29.82	0.14	6.78	27.0	45.3	44.20	0.16	6.13	19.6	48.6
7,8	31.56	0.10	6.65	25.0	36.3	37.86	0.20	6.65	25.0	54.0
9,10	31.56	0.13	6.26	28.0	39.1	37.86	0.18	6.84	27.9	66.8
ISOLEUCINE										
1,2	26.76	0.08	1.63	17.5	34.0	30.00	0.11	1.71	20.0	41.8
3,4	18.70	0.07	1.60	21.4	23.3	15.48	0.11	1.81	19.7	45.2
5,6	25.90	0.10	1.62	17.8	32.0	28.70	0.10	1.39	16.6	37.1
7,8	21.60	0.08	1.66	18.0	25.7	22.80	0.13	1.60	18.2	36.7
9,10	21.60	0.08	1.67	16.0	28.9	22.80	0.11	1.13	17.8	38.2
VALINE										
1,2	26.16	0.11	4.86	26.8	31.5	28.92	0.18	4.91	28.7	51.2
3,4	14.40	0.10	4.39	27.1	25.2	19.37	0.16	4.64	28.6	56.3
5,6	21.06	0.10	3.60	19.9	39.4	28.00	0.17	4.26	26.9	47.6
7,8	21.30	0.10	4.35	22.4	40.0	24.60	0.16	3.57	26.7	44.0
9,10	21.30	0.12	4.84	33.7	55.5	24.60	0.14	3.85	29.6	54.6
THREONINE										
1,2	16.80	0.27	4.21	16.8	36.0	22.80	0.59	4.37	24.6	55.2
3,4	10.14	0.20	4.05	18.5	45.2	12.90	0.36	4.07	20.1	49.1
5,6	17.16	0.32	3.94	19.0	36.8	19.90	0.43	3.71	21.1	54.0
7,8	15.00	0.26	4.13	20.8	32.6	19.44	0.47	3.91	21.6	51.3
9,10	15.00	0.28	3.85	21.7	35.6	19.44	0.44	4.02	21.0	60.7

(See footnotes under Table I-A.)

was accompanied only by a slight rise in the total methionine content of plasma in both subjects.

Individual Free Amino Acids in Plasma

The free amino acid content of plasma fluctuated slightly but did not parallel the varying intakes. Free methionine and free lysine increased minimally in both individuals after the addition of crystalline methionine to the diet; the concentrations of the other six amino acids did not vary significantly.

Combined Non-Precipitable Amino Acids in Plasma

Seven of the eight amino acids were consistently present, to some extent, in a non-precipitable "bound" form, *i.e.*, not precipitated with tungstic acid, yet not available to the assay organisms for growth. Non-precipitable combined methionine was found only in minute quantities or was totally absent. The combined non-precipitable amino acids in plasma varied in a random manner in both subjects. The addition of crystalline methionine was accompanied by slight increases in the levels of methionine and lysine only.

Amino Acid Output in Urine

The output of amino acids in the urine, as determined microbiologically, was very small (Tables

IA and B). The pattern of relative rates of excretion for the various amino acids was not directly related to the amounts taken in the food. Thus, the outputs of leucine and arginine were similar, although the intake of arginine was much smaller. The excretion of threonine exceeded that of valine although its intake was less. Histidine was excreted proportionately in far greater quantities than the other amino acids, averaging for the two subjects approximately 13 per cent of the intake. There appeared to be no correlation between the quantities of amino acids in the urine and the volume of urine or the output of urea nitrogen.

The fluctuations in excretion of histidine and threonine apparently paralleled the varying intakes. However, the outputs of methionine (periods 1-8), lysine, arginine, leucine, isoleucine and valine remained relatively constant. The quantity of methionine in the urine increased when 1.0 gm. of crystalline DL-methionine was added daily to a diet containing adequate amounts of this amino acid; however, the increment approximated only 0.5 per cent of the added methionine in J. D. and 0.7 per cent in R. G. H. The excretion of the other amino acids did not change significantly during the periods of methionine supplementation.

Subject 1 remained in approximate nitrogen

balance, and Subject 2, in positive nitrogen balance throughout the study (Table II). Retention of nitrogen increased markedly during periods 5 and 6, when the intake of protein was increased. The excretion of urea nitrogen, as expected, paralleled the quantity of nitrogen ingested. The small changes in output of free ammonia nitrogen did not appear significant.

Effect of Low Intake of Amino Acids

The effect of a markedly reduced intake of amino acids was investigated in Subject 2 during two additional periods, not included in the tables. In (a) the source of protein was a solution of peptone treated with 3 per cent hydrogen peroxide; the quantity of methionine ingested during the six-day period was decreased to 2.3 gms. In (b) the peptone solution was treated with 30 per cent hydrogen peroxide, the total intake of methionine being further reduced to 0.90 gm. In both periods, 1.25 gms. each of DL-tryptophane, L-tryosine, and L-cystine were added daily to compensate for the destruction of these amino acids by the peroxide. No effort was made to maintain the other amino acids at their original levels. The reduction in the intake of methionine in period (b) to 7.4 per cent of the subject's previous average intake was not

accompanied by a significant reduction in the methionine content of hydrolyzed whole plasma; however, the levels of free methionine decreased markedly. Although the intakes of the remaining amino acids, with the exception of arginine, also were considerably lower than in any of the previous periods, the concentrations of these seven amino acids in whole plasma likewise did not change. However, free lysine, histidine, leucine, isoleucine, valine and threonine decreased significantly; the concentration of free arginine did not vary. The combined non-precipitable levels in plasma did not diminish during the administration of the peptone diet. Supplementation of this regimen with 1.0 gm. of DL-methionine daily was not accompanied by significant changes in the concentrations of the eight amino acids in hydrolyzed whole plasma, or in the free amino acid levels. Non-precipitable combined methionine, lysine, arginine, and threonine increased slightly. The six-day output of methionine in the urine decreased from 0.108 gm. in period (a) to 0.04 gm. in period (b); the latter value, however, was smaller by only 27 per cent than the values obtained during the ingestion of much larger amounts of methionine. Of particular interest, perhaps, was the increased excretion of lysine, leucine, isoleucine,

TABLE II

Nitrogen balances and excretion of urea and free ammonia nitrogen in subjects J. D. and R. G. H.

Periods	Nitrogen Intake per 6-Day Period (gm.)	Balance per Period (gm.)	Urea Nitrogen in Urine	Free Ammonia Nitrogen in Urine (gm.)
Subject 1				
1,2	69.12	- 0.9	52.74	3.80
3,4	45.90	- 2.51	32.74	3.62
5,6	79.56	+12.04	51.53	5.31
7,8	70.32	+ 7.48	45.10	6.48
9,10	70.88	+ 1.20	51.15	4.61
Subject 2				
1,2	93.0	+ 6.6	66.10	4.24
3,4	57.48	+ 3.17	36.20	3.38
5,6	91.68	+ 9.48	62.70	4.38
7,8	80.34	+ 3.62	56.00	4.14
9,10	80.90	+ 2.92	55.65	4.58

0.56 gm. N in periods 9 and 10 in form of added crystalline methionine.
All values are averages of data for two successive six-day periods.

valine and threonine, the intakes of which had been reduced markedly. The output of arginine also rose although the quantity ingested was unchanged. The excretion of histidine diminished by 24 per cent, whereas the intake had been lowered by 84 per cent. During the same period (*b*) the output of urea nitrogen increased and the nitrogen balance became distinctly negative.

COMMENT

Plasma

The microbiological values in whole plasma for the eight amino acids are, with the exception of leucine, within the range listed for serum proteins by Block and Bolling (21). The data for leucine reported by the latter investigators were obtained by the oxidative method of Fromegat which, according to Hier *et al.* (22), yields values higher than are obtained by microbiological assay. The fasting whole plasma level of methionine averaged approximately 0.64 mg. per cent as compared with 0.73 mg. per cent, noted by Kinsell and his associates (23). The present data for free amino acids in plasma agree with the findings of Hier and Bergeim (24) in a group of 33 normal persons, and with the results for valine and histidine reported by Ackermann *et al.* (25). The individual amino acid values for the two subjects of this study were similar.

The whole plasma levels of lysine, arginine, histidine, leucine, isoleucine, valine and threonine did not fluctuate significantly although varying amounts were ingested. However, the methionine content of whole plasma apparently paralleled the varying intakes of this amino acid. Non-precipitable methionine accounted for only a minute fraction of these changes. Assuming that the constituent amino acid composition of plasma proteins remains constant, it may be postulated that a portion of methionine in plasma is attached to the surface of plasma proteins, is precipitable with the proteins by tungstic acid, yet does not constitute an integral component of the protein molecule. The concept of serum protein complexes has been discussed by various investigators (26-29). However, more data obviously are necessary to determine the significance of the fluctuations observed with methionine and to establish the validity of this hypothesis.

The effect of increased ingestion of one amino acid upon the individual levels of other amino acids

in the blood has been studied only occasionally. Hier (30) found that higher concentrations of free leucine, isoleucine and methionine in the blood of dogs, produced by feeding large amounts of the respective amino acids, were accompanied by decreases in the levels of certain other amino acids. However, the elevation in methionine was associated with a rise in the concentration of lysine. In the present study also, the increase in methionine, produced by the addition of crystalline DL-methionine to the diet, was accompanied by slight rises in the levels of free and combined non-precipitable lysine. The averaged data do not indicate the magnitude of the increase which resulted during the second period of methionine supplementation; the significance of these changes, however, remains to be determined.

Urine

The microbiological values obtained for total methionine, lysine, arginine, histidine, leucine, isoleucine, valine and threonine in the urine are within the ranges reported by Dunn and his associates (31), Woodson *et al.* (32), and by Eckhardt and Davidson (33).

Amino acid excretion was extremely small and relatively constant in relation to the amounts ingested. Eckhardt and Davidson similarly found no significant change in the quantity of amino acids excreted by a normal subject given varying amounts of protein. Although the output of methionine increased when the diet was supplemented daily by 1.0 gm. of the crystalline DL form, the increment for both subjects averaged only 0.6 per cent of the added methionine. Harper, Kinsell and Barton (34) noted a low output of methionine following its intravenous administration. Similar observations have been made by other investigators with respect to amino acid nitrogen (35).

It is to be noted that the number of calories was maintained at a constant level by adjustment of the carbohydrate and fat content of the diet. The data of Woodson *et al.* (32) have been interpreted as indicating that the caloric intake does not influence significantly the excretion of amino acids. However, this point has not been established conclusively, in our opinion. Until additional evidence is obtained, it would appear desirable, in studies of amino acid excretion, to control the caloric content of the diet. Preliminary experiments in our

laboratory suggest that the caloric intake may play a role in the urinary excretion of amino acids.

A small percentage of the amino acids in urine, as measured in this study, may have been derived from protein-like materials; Hanke (36) has reported the presence of a crystalline albumose in normal human urine which was excreted at a rate of approximately 50 mg. per day. Analysis of the amino acid composition of a sample of this material indicates, however, that less than 20 per cent of the total amino acids in urine may have been present in this form.

The increased excretion of amino acids in the urine of Subject 2 during the administration of an oxidized solution of peptone is of considerable interest. The oxidation obviously not only reduced the amino acid content, but also apparently disturbed the proper proportions of amino acids; the possible development of a toxic factor cannot be excluded. In this connection, Sauerblich, Pearce and Baumann (37) noted a higher excretion of "free" amino acids in the urine of mice fed diets containing protein of low biological value than among mice given proteins of ordinary or high biological value.

Both subjects remained in approximate nitrogen balance even when fed a moderately low protein diet. Nevertheless, nitrogen retention rose markedly when the intake of protein was increased. This finding suggests, perhaps, that measurements of nitrogen balance alone, at least for short periods of time, may not reveal minimally inadequate intakes of protein.

SUMMARY AND CONCLUSIONS

1. The amino acids methionine, lysine, arginine, histidine, leucine, isoleucine, valine and threonine were measured microbiologically in the food, plasma and urine of two normal adult men fed a variety of diets. In plasma, analyses were performed on acid-hydrolyzed whole plasma, tungstic acid filtrates (free amino acids), and on acid-hydrolyzed tungstic acid filtrates (free plus combined non-precipitable amino acids).

2. The fasting amino acid content of plasma, in general, did not fluctuate significantly despite considerable variation in the quantity of amino acids ingested in the food. Parallel fluctuations in the plasma levels and the amino acid intakes were observed in both subjects only for total methionine.

3. The output of amino acids in the urine was small, averaging 2.5 per cent of the intake. The pattern of relative rates of excretion for the various amino acids was not directly correlated with the respective individual amounts taken in the food.

4. Excretion of six of the eight amino acids was relatively constant despite considerable variations in intake. The outputs of histidine and threonine fluctuated slightly in relation to the varying intakes.

5. The addition of crystalline DL-methionine to a diet adequate in this amino acid was accompanied by increases in the whole plasma levels of methionine and of free and combined non-precipitable methionine and lysine. The urinary output of methionine increased slightly; no significant changes occurred in the excretion of the other amino acids.

6. In one subject the oral administration of a peptone solution treated with hydrogen peroxide and containing markedly reduced quantities of methionine, lysine, histidine, leucine, isoleucine, valine and threonine, was accompanied by definite decreases in the free levels of these amino acids. During the same periods, the amino acid outputs in the urine increased considerably.

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FACTORS IN THE TREATMENT OF LAENNEC'S CIRRHOSIS. I. CLINICAL AND HISTOLOGICAL CHANGES OBSERVED DURING A CONTROL PERIOD OF BED-REST, ALCOHOL WITH- DRAWAL, AND A MINIMAL BASIC DIET^{1,2}

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Fatty infiltration and cirrhosis of the liver can be produced experimentally in animals by defective diets, and can be prevented or cured by supplements of protein or lipotropes (1-10). Clinical observations suggest that dietary deficiency may also play a role in the pathogenesis of Laennec's cirrhosis in man (11), and a number of investigators have reported that dietary supplements of protein, vitamins and lipotropes affect the course of the disease favorably (11-18).

While it is generally agreed that these dietary supplements are of therapeutic value in human cirrhosis, it is still not known whether all are essential, whether their effects are due to a specific action on the liver, or to a more general one on the associated malnutrition, and whether the results of treatment are influenced by non-dietary factors. The difficulty in obtaining suitable controls, and the tendency to use multiple therapeutic measures simultaneously have delayed the solution of these problems.

The results of dietary therapy in cirrhosis are usually evaluated in one of two ways. In the first, longevity statistics are used as the criterion for comparing treated and untreated groups, the latter having usually been observed some years previously (11, 15, 16). In the other, the effects of various dietary measures are compared either in the same patient or in small groups, using serial clinical, functional and, in some instances, histological observations as the basis for comparison (13, 14, 17, 18). There are valid objections to both methods, but serial observations during alternating

courses of therapy in the same subject appear to offer the most practical means for conducting experiments under controlled conditions.

The natural history of Laennec's cirrhosis and its clinical and morphological manifestations are so variable that observations based on small groups are difficult to interpret, even when the rigid criteria of Volwiler (18) are met. Longevity statistics are even more difficult to analyze, especially when the controls have been observed some years before the experimental subjects. The difficulty in obtaining complete and reliable follow-up data in a large series, the relatively high mortality from unrelated diseases and the changing outlook in the treatment of the two most common serious complications, hemorrhage and infection, are some of the factors which distort the results. The importance of the experimental method and the type of control employed is well illustrated by the conflicting reports of Patek and Post (11) and Wade (19) on the effects of dietary therapy in cirrhosis, and of Volwiler (18) and Beams (14) on the effects of lipotropic substances.

Alcohol withdrawal and bed-rest are often factors in therapeutic experiments in cirrhosis, but are seldom taken into account in evaluating the results. The position of alcohol in the pathogenesis of Laennec's cirrhosis is still uncertain. Alcoholism is now generally regarded as an important factor conditioning malnutrition, but is not thought to have any more direct effect on the liver (11, 18). While there is no good evidence that alcohol acts directly as an hepatotoxin, several experiments suggest that it can play a role in the production of cirrhosis independent of its effects on food intake (20).

The importance of bed-rest has been stressed in infectious hepatitis (21), and may well be of

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significance in the treatment of Laennec's cirrhosis (18).

During the past year we have been investigating the effects of dietary supplements of choline and protein in the treatment of Laennec's cirrhosis. As a preliminary each subject has been observed during a control period of three to seven weeks under conditions of complete bed-rest, alcohol withdrawal and a basic diet. Such striking changes have occurred during this period that the original experiments designed to test the efficacy of dietary supplements have had to be revised and then delayed. The following is a report on the clinical, functional and histological changes observed during the control period in 14 subjects.

MATERIAL AND METHODS

The 14 subjects chosen for investigation were routine admissions to the wards of the New Haven Hospital and the Newington Veterans Hospital. With one exception all had moderate to advanced Laennec's cirrhosis and

were admitted because of symptoms referable to it (Table I, Figure 1). Case 9v had a severe fatty infiltration of the liver without fibrosis and was admitted because of a small hemoptysis secondary to bronchiectasis. This patient was included in the series on the assumption that fatty infiltration of this type represents an early stage of Laennec's cirrhosis (22).

There was no selection of cases, all proved cases of Laennec's cirrhosis admitted to the wards of both hospitals being included in the study, provided they were able to stay in the hospital for a minimum of three weeks, were able to take food by mouth, and had not received intensive treatment before admission. All treatment prior to the beginning of the control period is indicated in Table II.

Each of the subjects had the liver function tests outlined below at one- or two-week intervals, and a liver biopsy by Vim-Silverman needle at the beginning and end of each experimental period. The two biopsy sites were never more than a centimeter apart. The site chosen depended on the size and position of the liver, and was epigastric in Cases 1, 2v, 3, 4, 6, 8v, 9v, 11v and 12, right subcostal in Cases 5v, 7v and 13v, and right intercostal in Cases 10 and 14.

TABLE I
Summary of findings on admission to hospital

Case No.	1	2v	3	4	5v	6	7v	8v	9v	10	11v	12	13v	14
Age, years	36	40	53	42	35	64	43	36	40	65	55	48	35	48
Sex	M	M	F	F	M	M	M	M	M	F	M	M	M	M
Alcoholism	3+	3+	71+	2+	3+	2+	2+	2+	3+	0	3+	3+	2+	3+
Inadequate diet	2+	2+	2+	2+	0	0	0	0	2+	0	2+	3+	3+	3+
Symptoms, duration														
jaundice	8d.	7d.	0	0	0	1m.	?	4d.	0	2d.	1w.	0	1m.	0
ascites	10d.	2w.	10w.	3½m.	2w.	0	12d.	4d.	0	1m.	4d.	0	0	9w.
edema	0	7d.	?	1½y.	2m.	2m.	12d.	0	0	0	0	3w.	4d.	4w.
dyspnea	0	7d.	10w.	2w.	0	1y.	0	0	0	1m.	4d.	0	1m.	0
dyspepsia and anorexia	0	0	10w.	2w.	2w.	3m.	7m.	1m.	3m.	1m.	3w.	2m.	4m.	9w.
hematemesis	0	0	0	0	10d.	0	0	0	0	0	74m.	0	0	0
diarrhea	8d.	7m.	10w.	3½m.	3w.	2m.	0	1m.	0	0	0	0	1m.	0
wt. loss, lbs.	37	?	14	14	8	30	8	?	?	?	?	17	33	?
duration	7y.	?	10w.	1y.	2w.	3m.	2y.	1m.	—	1m.	4m.	2m.	4m.	?
Physical findings, severity														
malnutrition	0	3+	2+	2+	0	0	1+	2+	0	0	2+	3+	3+	3+
jaundice	2+	1+	0	0	1+	1+	1+	1+	0	1+	3+	0	1+	0
ascites	1+	3+	2+	1+	1+	0	2+	2+	0	2+	71+	0	0	3+
edema	0	3+	1+	0	0	1+	1+	1+	0	0	0	2+	2+	2+
pleural effusion	0	2+	1+	0	0	0	1+	1+	0	1+	0	0	0	0
spider nevi	1+	1+	1+	1+	0	0	3+	1+	0	1+	0	0	0	3+
liver														
hepatomegaly	5f	2f	3f	5f	8f	7f	5f	3f	4f	0	5f	4f	4f	0
induration	3+	2+	2+	2+	2+	2+	2+	2+	0	—	2+	2+	2+	—
splenomegaly	1+	0	0	1+	0	0	0	0	0	2+	0	0	1+	0
venous collaterals	2+	1+	1+	2+	1+	2+	0	2+	0	2+	2+	0	0	1+
esoph. varices (x-ray)	+	0	—	0	0	0	0	+	0	0	0	—	—	0
fever (max.)	0	101.5	101	0	100	102	100.5	100.8	0	102	100	0	100	101.4
Complications	0	a.	b.	c.	0	0	0	0	d.	e.	0	f.	g.	h.

Symbols: 0 = absent, 1+ = mild, 2+ = moderate, 3+ = severe; M = male, F = female; d. = days, w. = weeks, m. = months, y. = years; f = fingers breadth below right costal margin.

Complications: a. = mild peripheral neuritis, mild ulcerative colitis; b. mild glossitis; c. asthma; d. bronchiectasis and small hemoptysis (cause of admission); e. peritonitis, RLQ abscess drained 2 mos. later, source not determined; f. mild glossitis and painful legs, no signs of neuritis; g. mild peripheral neuritis; h. marked cheilosis, glossitis, unilateral gynecomastia.

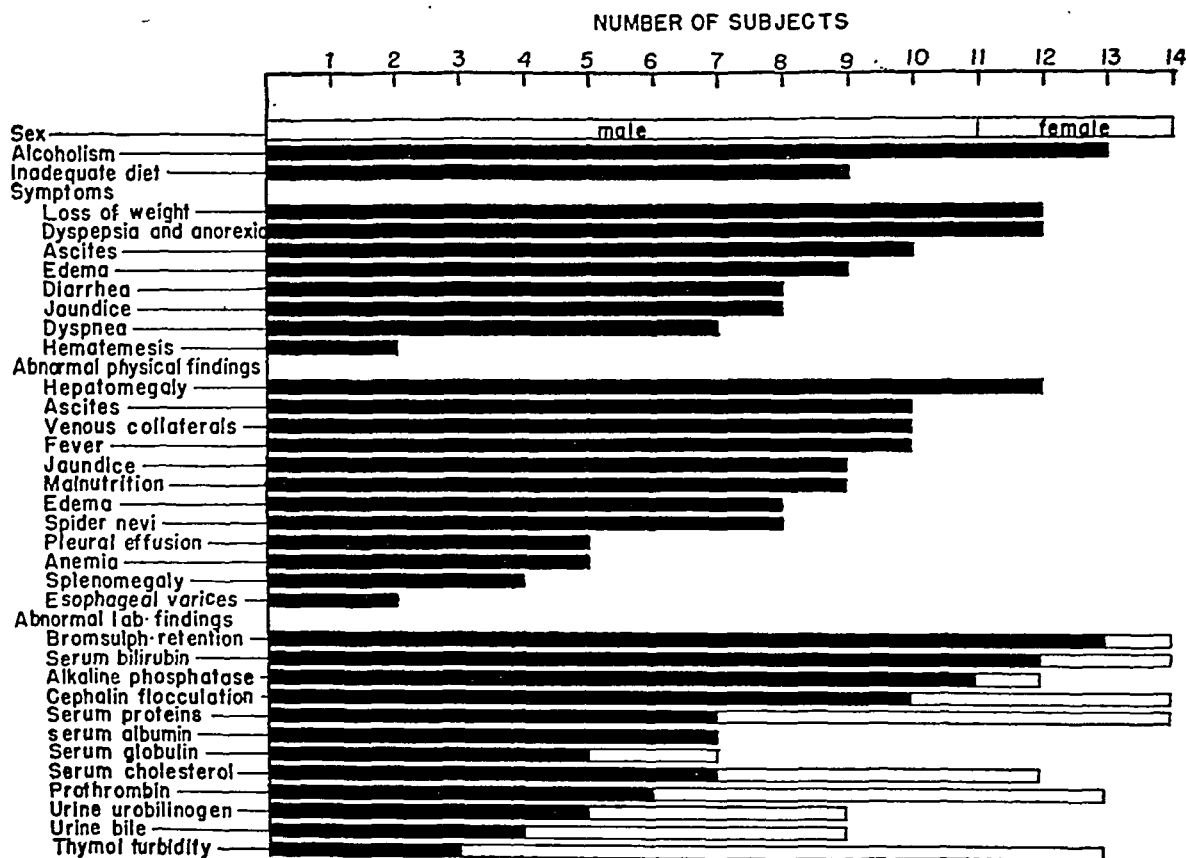


FIG. 1. CLINICAL STATUS OF SUBJECTS ON ADMISSION TO THE HOSPITAL

The length of the solid bars indicates the number of abnormal findings. The open bars indicate the total number of tests performed.

Esophageal varices demonstrated by X-ray.

Abnormal laboratory values: BSP retention $> 5\%$ in 45 minutes, $> 10\%$ in 30 minutes; serum bilirubin > 1.0 mg.%; alkaline phosphatase > 4.0 Bodansky or > 12 King-Armstrong units; cephalin-cholesterol flocculation $> 1+$; serum proteins < 6.5 gm.%; serum albumin < 3.0 gm.%; serum cholesterol < 150 or > 250 mg.%; prothrombin $< 70\%$; urine urobilinogen $> 1:10$; urine bile $1+$; thymol turbidity > 4.0 units.

The clinical studies and biopsies were performed by one of us (G. K.), while the other (R. Y.), who had no contact with the subjects nor any knowledge of their clinical status, reviewed all the sections of biopsy material at the end of the experiment, and evaluated them as outlined in Table III. Biopsy material was fixed in 10% formalin in absolute alcohol, cut at 5 microns and stained with Best's carmine and eosin and hematoxylin. Half the sections were also stained for reticulum and connective tissue with Laidlaw's stain.

The laboratory studies were carried out by identical methods in the two hospitals, with the exceptions noted, and included serum bilirubin (23), cephalin-cholesterol flocculation (24), thymol turbidity (25), bromsulphalein retention (26), serum alkaline phosphatase (27), prothrombin (28), urine bilirubin (29) and urine urobilinogen (30). Serum proteins were determined by the macro-Kjeldahl method at New Haven and the Phillips' falling drop method (31) at Newington. Serum cholesterol was

determined by the method of Peters and Man (32) at New Haven and by the Bloor method (33) at Newington. In a few instances the serum alkaline phosphatase was carried out by the King-Armstrong method (34) at New Haven; these values are marked "K" in the tables.

The experimental diet was started within a day or two of the patient's admission to the hospital, with the exceptions noted in Table II, and the patient was kept at bed-rest throughout the period of observation. The diet contained 1 gm. of protein per kilogram of body weight at the time of admission, allowance being made for accumulated fluid, 100 gm. of fat, and enough carbohydrate to raise the total intake to 30 calories per kilogram. Neither vitamins nor other dietary supplements were allowed, with the exception that patients with very low prothrombin levels were given vitamin K.

The control period (Period I) varied in duration from 21 to 49 days. Six of 14 subjects, who were available for further study under the same conditions, received

TABLE II. *Clinical and laboratory observations at the beginning and end of the control period.*

Case	Duration of period	Diet	Clinical								1 min. bili-rubin mg. %	Total bili-rubin mg. %
			Weight kg.	Para-centeses (number)	Ascites	Jaundice	Edema	Hepato-megaly	Fever max.	Symp-toms		
1	22	P70 F100 C250 32 calories per kg. All eaten.	B. 69.0	0	1+	2+	0	5f	0	2+	2.41	10.20
		E. 65.5			2+	3+	1+	5f	102.5	1+	2.41	14.76
2v	28	P70 F100 C230 31 calories per kg. All eaten.	B. 68.2	0	2+	1+	3+	2f	101.5	3+	0.30	1.35
		E. 63.2			0	0	0	1f	0	1+	0.20	0.85
3	24	P60 F100 C180 36 calories per kg. All eaten.	B. 51.4	4.0 L. (1)	2+	0	1+	3f	101	2+	0.41	1.22
		E. 52.6			1+	0	0	3f	100	1+	0.07	0.61
4	21	P70 F100 C200 32 calories per kg. All eaten.	B. 61.4	0	1+	0	0	5f	0	2+	0.07	0.68
		E. 63.4			0	0	0	5f	0	0	0.24	0.68
5v	28	P93 F100 C357 29 calories per kg. All eaten.	B. 94.5	0	1+	1+	0	8f	100	2+	1.00	2.00
		E. 96.8			0	0	0	8f	100	1+	0.20	0.70
6	26	P70 F100 C250 24 calories per kg. All eaten.	B. 92.0	0	0	1+	1+	7f	102	2+	1.29	2.38
		E. 88.9			0	1+	?	6f	102	1+	0.68	2.38
7v	42	P85 F100 C300 30 calories per kg. All eaten.	B. 80.0	1.0 L. (1)	2+	1+	1+	5f	100.5	1+	0.30	1.85
		E. 75.5			0	0	0	5f	100	0	0.20	1.00
8v	35	P65 F100 C200 30 calories per kg. Eaten poorly for half the period.	B. 65.5	13.9 L. (3)	2+	1+	1+	3f	100.6	3+	1.50	3.10
		E. 58.0			2+	1+	1+	3f	100.4	2+	1.55	3.60
9v	42	P70 F100 C230 29 calories per kg. All eaten.	B. 72.7	0	0	0	0	4f	0	1+	0.03	1.55
		E. 72.7			0	0	0	0	0	0	0.20	0.85
10	32	P85 F100 C250 27 calories per kg. Poorly taken.	B. 83.2	4.0 L. (2)	2+	1+	0	0	102	2+	0.41	1.70
		E. 76.5			2+	1+	0	0	102	2+	0.27	1.38
11v	49	P71 F100 C234 29 calories per kg. All eaten.	B. 72.3	0	1+	3+	0	5f	100.2	3+	12.00	17.10
		E. 68.6			0	1+	0	2f	0	0	1.25	2.70
12	41	P55 F80 C515 60 calories per kg. Measured intake from 27-60 calories per kg.	B. 55.9	0	0	0	2+	4f	0	3+	0.34	0.48
		E. 57.3			0	0	0	2f	0	1+	0.14	0.27
13v	38	P55 F100 C132 30 calories per kg. All eaten.	B. 55.5	0	0	1+	2+	4f	100	2+	1.25	2.00
		E. 55.9			0	0	0	4f	0	0	0.10	0.20
14	32	P70 F100 C230 30 calories per kg. Poorly taken last two weeks.	B. 69.5	18.7 L. (4)	3+	0	2+	0	100.8	3+	0.27	0.38
		E. 55.7			3+	1+	2+	0	101.4	3+	0.85	2.58

Symbols as in Table I. B = beginning, and E = end of control period.

* Cephalin-cholesterol flocculation: 24 hrs./48 hrs.

† Bromsulphalein retention after 5 mg. per kg. of dye intravenously: 30 min./45 min.

TABLE II (continued)

Case	Laboratory													Remarks
	Ceph. flocc.*	Bromsul-phalein retention†	Alk. phosphatase‡	Serum prot.	Serum alb.	Serum glob.	Total cholesterol	Free cholesterol	% free cholesterol§	Prothrombin	Urine bile	Urine urobilinogen	Thymol turbidity	
		%	units	gm. %	gm. %	gm. %	mg. %	mg. %		%			units	
1	1+/1+	51/	3.52	7.05	2.50	4.55	112	88	79	70	4+	1:10	4.0	Clinical and functional improvement until 14th day when pt. developed sore throat and fever, following which relapse occurred. Obviously lost tissue.
	0/0	—	2.20	5.84	2.77	3.07	146	99	68	62	4+	1:160	3.0	
2v	/3+	/28.5	7.70	7.44	—	—	134	—	—	35	tr.	—	5.5	Obviously lost tissue and wanted more food. During week before study received choline 4.0 gm. and methionine 4.0 gm. daily and had two paracenteses.
	/3+	/4.5	8.80	8.45	—	—	245	—	—	68	—	—	6.0	
3	0/0	42.9/	8.10	6.30	1.21	5.09	88	34	39	100	0	1:40	3.5	During week before study received high protein diet and had paracentesis of 5.7 L. Gained tissue on experimental diet.
	0/0	9.3/	5.28	6.98	2.15	4.83	168	51	33	—	0	0	3.0	
4	0/0	36/	11.43	7.02	2.77	4.25	196	63	32	100	1+	1:20	2.0	
	0/0	21.6/	17.00	6.20	2.58	3.62	—	—	—	—	0	1:20	2.0	
5v	/3+	26.5/	5.1	7.15	—	—	233	—	—	85	0	1+	6.5	During week before study received high protein diet, which was very poorly taken, and had paracentesis which yielded a small amount fluid.
	/3+	/6.0	1.2	7.80	—	—	198	—	—	51	—	—	3.0	
6	3+/4+	40/	—	5.60	1.35	4.26	—	—	—	55	—	—	6.5	Regular diet for five days before study.
	2+/4+	36/	—	6.77	1.37	5.40	—	—	—	—	—	—	20.0	
7v	/3+	/24	7.40	7.80	—	—	135	—	—	100	—	—	1.5	Paracentesis performed second day of study—no recurrence of fluid.
	/2+	/29	7.60	7.80	—	—	166	—	—	—	—	—	2.0	
8v	/4+	/49	6.6	5.10	—	—	176	—	—	51	3+	4+	2.0	During 17-day period before study received high protein diet and had paracentesis of 4.1 L. Severe hematemesis 13th day of study, requiring 5.0 L. of blood.
	/3+	—	5.3	5.20	—	—	186	—	—	—	—	—	3.0	
9v	/0	/10.5	—	7.40	—	—	205	—	—	—	0	—	2.5	
	/1+	/0	4.3	—	—	—	260	—	—	—	0	0	2.0	
10	1+/2+	32/	7.5k	5.63	2.83	2.80	100	59	59	70	—	1:80	1.0	Paracentesis (X2) of 9.16 L. and low fat diet during 11-day period before study. Signs of peritonitis, operation refused. Abscess drained two months later, initial site undetermined. Obviously lost tissue.
	2+/4+	40/	—	4.79	1.61	3.18	—	—	—	55	0	—	1.0	
11v	/3+	/36	13.6	6.30	—	—	460	—	—	50	—	—	4.0	
	/0	/24.5	7.0	7.80	—	—	360	—	—	65	—	—	3.0	
12	1+/2+	20/	13.4k	5.35	2.96	2.39	126	38	30	100	0	1:32	4.0	
	1+/2+	4/	8.1k	6.00	3.07	2.93	172	54	31	—	—	—	1.0	
13v	/3+	/0	6.9	6.3	—	—	308	—	—	63	—	—	1.00	Vitamin B complex parenterally first seven days of study. Gained tissue as edema subsided. Wanted more food.
	/2+	/0	4.2	7.8	—	—	268	—	—	50	—	—	3.75	
14	3+/4+	/45	19.5k	6.30	2.35	3.95	170	56	33	55	—	0	5.0	Clinical improvement first three weeks, then downhill course with increasing anorexia, ascites and weight loss.
	3+/4+	—	13.3k	5.79	1.38	4.41	—	—	—	55	1+	1:40	5.0	

† Bodansky units, except for values marked k. (King-Armstrong units).

§ Free cholesterol, per cent.

TABLE III
Histology of the liver at the beginning and end of the control period

Case	Fat infiltration			Pigment		Necrosis		Cellular inflt.		Pleomorphism	Fibrosis	Bile duct prolif.	Intra-lobular disorganization
	Degree	Droplet size	Distribution†	Intra-cellular	Bile thrombi	Hyalin	Focal	Cell type‡	Degree				
1 B.* E.	+++ +++	+++ +++	T T	++ ++	+++ ++++	+++ +++	0 0	L PLM	+ ++	+++ +++	++++ ++++	++ ++	+++ +++
2v B. E.	+++ +	+++ +++	T MC	0 0	0 ±	0 0	± ±	PLM PLME	+ +	+ ++	++++ ++++	++++ ++++	+++ +++
3 B. E.	++ +	++ +	T T	+ +	+ ±	0 0	0 0	LM LM	++ +	+ ++	++++ ++++	++++ ++++	+++ +++
4 B. E.	+++ ++	++ ++	T T	0 0	0 0	± +	0 0	LM LM	+ ++	+ +	++++ ++++	++++ ++++	+++ +++
5v B. E.	++++ ++++	++++ ++++	T T	+ +	+++ ++	0 0	0 0	LM LMP	+++ +++	++ +++	++++ ++++	+++ +++	+++ +++
6 B. E.	+++ ++	++ +++	T T	0 0	0 0	++ ++	0 0	LM LM	+ +	+++ +++	++++ ++++	++ ++	+++ +++
7v B. E.	++ ±	+ +	C C	± 0	0 0	0 0	++ ±	LMP LM	++ +	+++ ++	+++ ++	++ +	+++ +++
8v B. E.	++ +	++ ++	T T	0 0	+ 0	+++ +	0 0	PL PL	++ +	+++ ++	+++ ++	+++ ++	+++ +++
9v B. E.	+++ ++	+++ +++	T CM	± ±	+ +	0 0	+ 0	PL L	+ ±	++ +	0 ±	0 0	+++ ±
10 B. E.	++ +	+++ +++	T T	0 0	0 0	0 ±	0 0	L 0	++ 0	± ++	+++ +++	+++ ++	+++ +++
11v B. E.	+++ ++	+++ ++	T T	+ ±	+++ ±	0 0	± 0	P PM	+ +	± +	++ ++	++ ++	+++ ++
12 B. E.	+++ ++	+++ +++	T T	0 +	0 0	+ 0	0 0	ME ME	± ++	++ ++	++ ++	++ +	+++ +
13v B. E.	++ 0	++ 0	T 0	++ ++	+++ ++	0 0	0 0	0 L	0 ±	+ +	++ ++	++ ++	++ ++
14 B. E.	+ ±	++ +	C C	0 0	0 0	+++ +++	0 0	PLE PME	+ +	++ ++	+++ +++	+++ +++	+++ +++

* B. = beginning, and E. = end of control period.

† T = throughout lobule, C = central, M = mid-zonal.

L = lymphocytes, M = monocytes, P = polymorphonuclears, E = eosinophiles, P = plasma cells.

supplements of choline (Period II) and protein (Period III), in addition to the basic diet, following completion of the control period.

The present report is concerned primarily with observations made during Period I. The experiments dealing with the effects of dietary supplements are still in progress and will be discussed only briefly in connection with the interpretation of the effects of the control period.

RESULTS

Status on Admission. The clinical and laboratory findings are those usually seen in moderate to advanced Laennec's cirrhosis (Table I, Figure 1). The preponderance of males can be ascribed, in part at least, to the almost exclusively male population of the Veterans Hospital, where half the subjects were studied. Alcoholism and poor diet appeared to be important features in the past history, although reliable quantitative data could not be obtained. The high incidence of jaundice and ascites testifies to the severity of

the disease in most of the cases, and in only one (Case 9v) could the liver be regarded as being in a state of "compensation."

Hepatic enlargement was present in all but two subjects (Cases 10 and 14) and may have been related to the degree of fat infiltration noted histologically, although the causal relationship between the two is by no means certain (35).

The impairment of hepatic function demonstrated by laboratory methods was reasonably well correlated with the clinical status, the most significant findings being an elevation of the serum bilirubin level, increased bromsulphalein retention and a decrease in serum albumin. The serum cholesterol findings were of interest in that the total level was decreased in five, and increased in two. Of the six subjects in whom the cholesterol was fractionated, only two showed a pathological increase in the free:total ratio. The level of serum cholesterol and its fractions did not appear to be

related to the clinical, functional or histologic status of the liver, nor to its responsiveness to treatment.

The histologic findings were typical of Laennec's cirrhosis. Fatty infiltration of the parenchyma, fibrosis, periportal cellular infiltration and distortion of the architecture of the lobules predominated. Case 9v showed maximal fatty infiltration but no fibrosis, while Case 14 with the most marked fibrosis, showed little fat. No mitotic figures were seen, but the parenchymal cells frequently exhibited variations in size and staining qualities with many large binucleate cells and hyperchromatic nuclei, which were interpreted as probable signs of regeneration. Hyaline necrosis occurred much less frequently than in other studies of biopsy material in Laennec's cirrhosis, and may be related to our use of formalin-absolute alcohol fixative instead of Zenker's fluid (18). The individual histologic find-

ings were poorly correlated with specific tests of liver function, or with particular clinical manifestations, and their severity reflected only in a very general way the severity of the disease clinically. In Volwiler's experience there has been a similar poor correlation of histologic, functional and clinical findings (18).

Changes Observed at the end of the Control Period. Improvement in the clinical, functional and morphological status of the liver occurred in a significant number of the subjects (Tables II and III, Figure 2). The degree of improvement varied from case to case, and was not uniform by all criteria, but it appeared to be roughly correlated with the initial severity of the fat infiltration, an observation previously noted by others (18, 36). Thus, striking improvement occurred in Case 9v with the most marked fat infiltration (Figure 3),

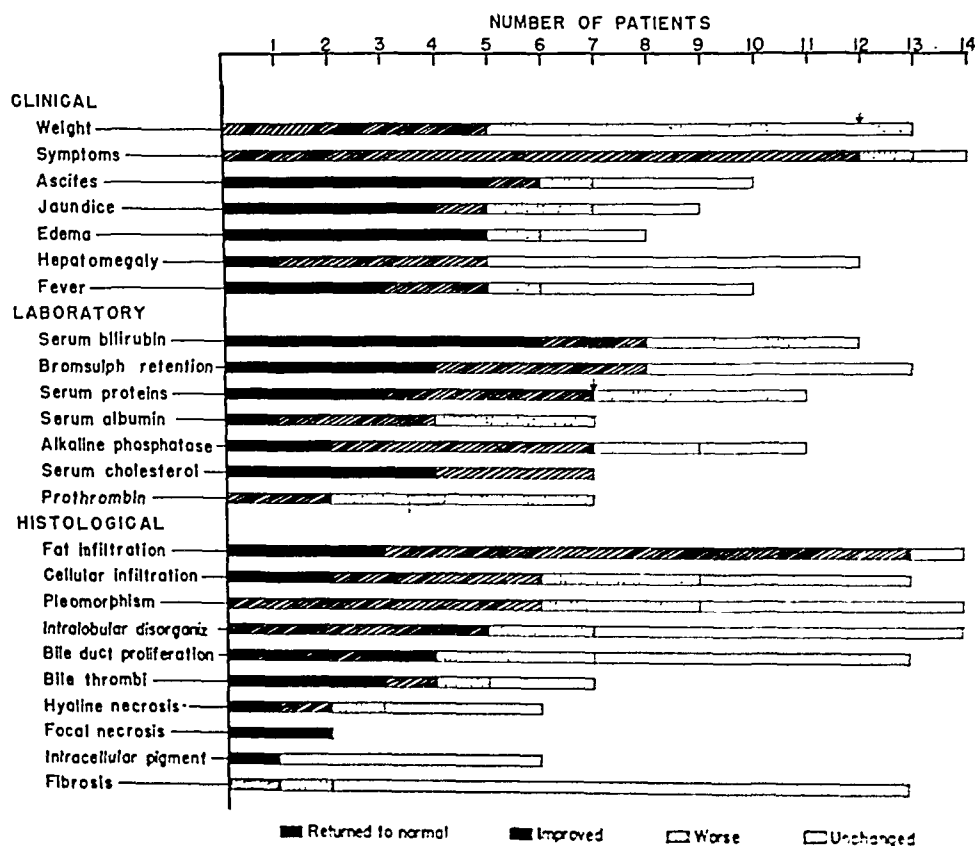


FIG. 2. CHANGES NOTED DURING CONTROL PERIOD

The total length of the bars indicate the number of abnormal findings at the beginning of the control period, except in the case of weight and serum proteins where the original number of abnormal values is indicated by an arrow.

In the histological data \pm values were regarded as within normal limits. An increase in pleomorphism was regarded as a sign of regeneration and improvement.

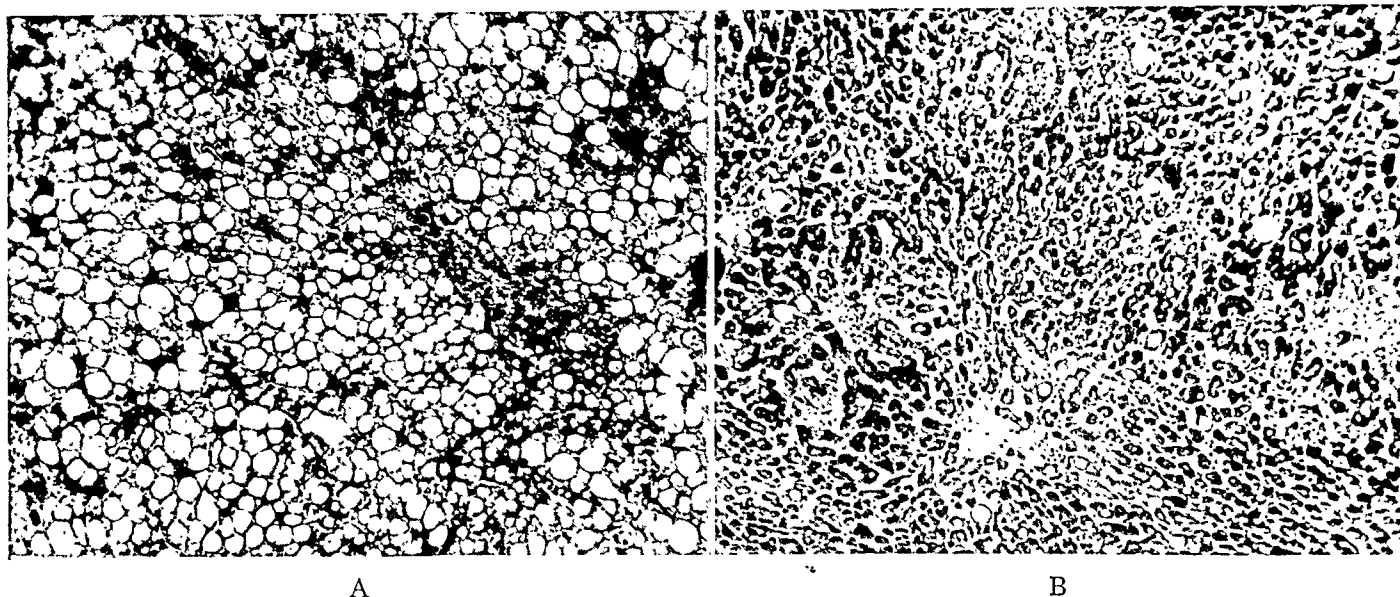


FIG. 3. LIVER BIOPSY, CASE 9v

A. Before treatment, showing marked fat infiltration without significant fibrosis, distortion of the lobular architecture, and small compressed parenchymal cells (hematoxylin and eosin $\times 150$).

B. Forty-two days on control regime, showing almost complete loss of fat and restoration of normal lobular architecture and normal liver cords (Best's carmine $\times 150$). The liver had returned to normal size, and function tests were within normal limits at this time.

while the clinical course was rapidly and progressively downhill in Case 14 with minimal fat infiltration, even after the basic regime was replaced by a high protein, high caloric diet supplemented with choline and parenteral vitamins, liver extract and serum albumin.

The appearance of a complicating infection affected the course of the disease unfavorably in at least two subjects. Case 1 showed subjective and objective improvement during the first two weeks of the control period, but became more jaundiced, developed edema and exhibited an increase in ascites and a decrease in his serum proteins concomitant with the onset of a severe penicillin-resistant streptococcal sore throat in the third week. Unfortunately the control period was terminated at this point and choline was added to the basic regime. As the infection subsided there was clinical and functional improvement again. The cirrhosis was complicated by an unexplained suppurative peritonitis in Case 10, and there was little improvement during the control period except for a slight fall in the serum bilirubin level, and a decrease in fat infiltration histologically. Significant improvement did not occur until the infection was brought under control by surgical drainage of the abdomen combined with chemotherapy.

Subjective improvement in the initial symptoms

was observed in all the cases, except 10 and 14. This was accompanied by a decrease or loss of ascites, edema, jaundice, hepatomegaly and fever in more than a third of the group. Ascites subsided completely in five cases and decreased in one. The ascites was sufficiently severe in these to have required paracentesis in four.

Clinical improvement was usually accompanied by evidence of improved hepatic function, although there were several notable exceptions. Approximately half the subjects exhibited a fall in serum bilirubin and bromsulphalein retention and a rise in serum protein and albumin. In a significant number these values returned to normal. There was a fall in serum protein despite improvement by other criteria in two instances. In Case 4 the fall was due to a decrease in globulin without a significant change in the albumin fraction. In Case 10 both the total protein and albumin fraction fell despite morphologic improvement and a slight decrease in serum bilirubin. The fall in serum albumin in this instance may have been related to a loss by paracentesis and to an inadequate protein intake in the face of infection, rather than to a deterioration of hepatic function.

The changes in serum cholesterol were of interest. In every instance where the level was abnormal there was a rise or fall toward the nor-

mal range, depending on whether the initial value was low or high. The free: total cholesterol ratio was followed in only three subjects. Initially the ratio was abnormally high in two and normal in the third. It fell to normal in one, declined slightly in the second and did not change significantly in the third.

The changes in cephalin-cholesterol flocculation and thymol turbidity were not considered significant. The prothrombin level fell in five cases, but the fall was significant in only one instance (Case 5v). Only Case 2v showed a significant increase in prothrombin.

The most striking change observed histologically

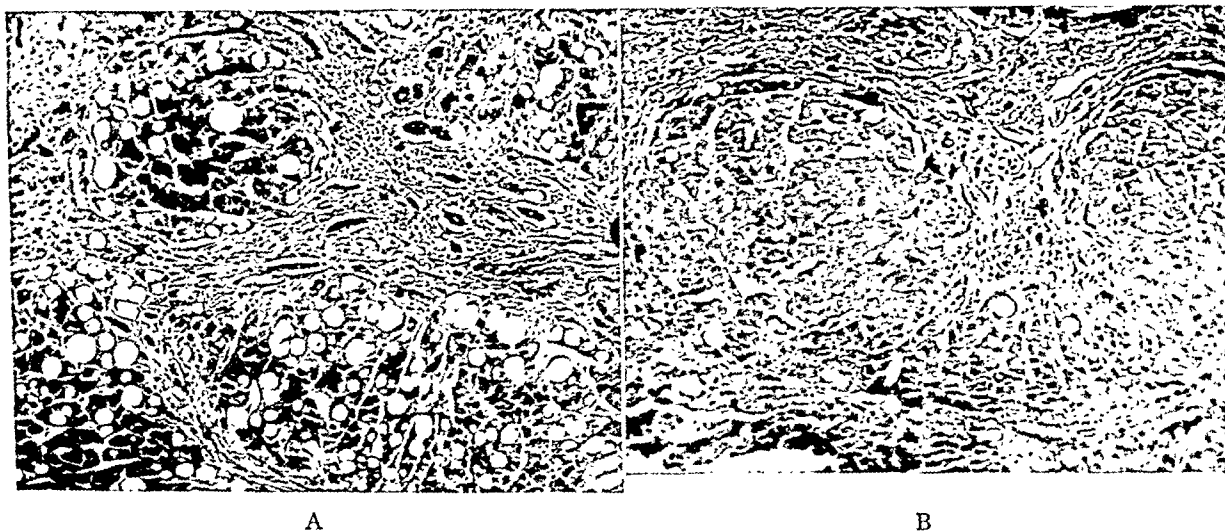


FIG. 4. LIVER BIOPSY, CASE 2v

A. Before treatment, showing marked fibrosis, moderate fat infiltration, disruption of the normal lobular architecture, and bile duct proliferation (hematoxylin and eosin $\times 150$).

B. Twenty-eight days on control regime, showing a significant decrease in fat and larger, more normal appearing liver cords (hematoxylin and eosin $\times 150$). The liver had decreased in size, and function tests were within normal limits at this time.

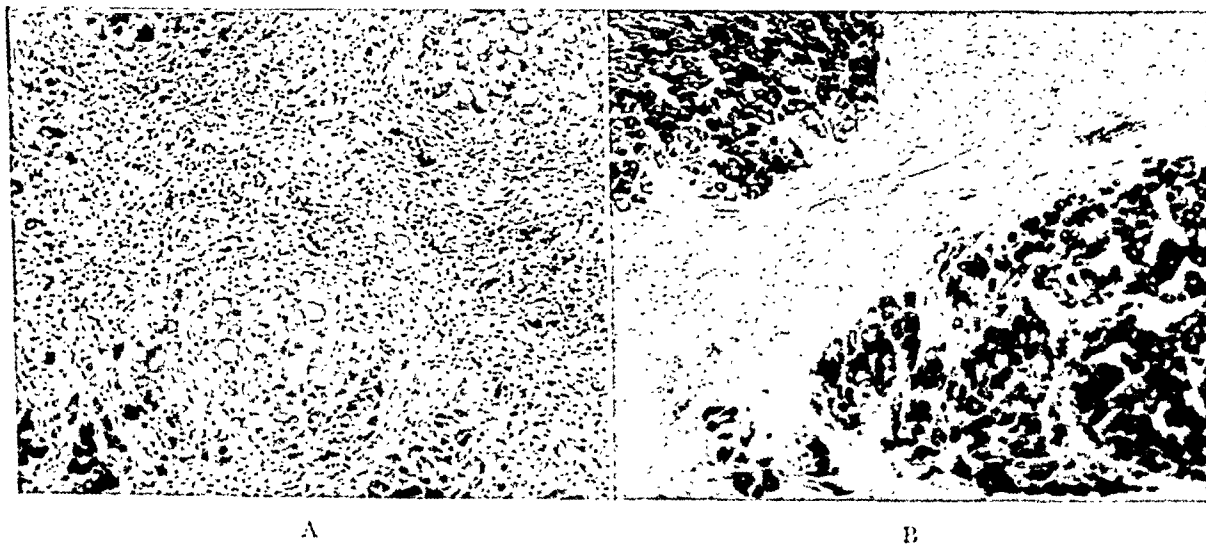


FIG. 5. LIVER BIOPSY, CASE 14

A. Before treatment, showing very extensive fibrosis, marked disruption of the lobular architecture, bile duct proliferation, and slight fat infiltration (hematoxylin and eosin $\times 150$).

B. Thirty-two days on control regime, showing complete loss of fat despite obvious deterioration of clinical and functional status. The apparent shrinkage of the liver cords is due to the stain employed (Best's carmalum $\times 150$).

was a decrease in fat infiltration which occurred in all but one subject (Figures 3-5). The fat actually disappeared in three instances, including Case 14 whose clinical and functional status deteriorated (Figure 5). Further evidence of healing was found in the increased regeneration of parenchymal cells, the decrease in periportal cellular infiltration and the improvement in intralobular organization, which occurred in almost half the cases. The other signs of histologic improvement noted in Table III occurred less frequently. There was no significant decrease in fibrosis in any of the cases.

The validity of the method of sampling the liver by needle biopsy is obviously open to some question, but the uniform trend of the changes observed suggests that they were of significance.

COMMENT

It is apparent from these observations that significant improvement may occur in Laennec's cirrhosis under conditions of bed-rest, alcohol withdrawal, and a minimal basic diet, and that these factors must be taken into consideration in evaluating the specific therapeutic actions of dietary supplements. The results, however, do not warrant any definite conclusions regarding the relative efficacy of the basic diet and diets supplemented with protein or lipotropes, nor do they indicate which of the factors in the control regime was responsible for the apparent improvement observed.

The six subjects who received supplements of choline in Period II and of protein in Period III, under continued conditions of bed-rest and alcohol restriction, showed progressive improvement clinically and histologically. Since there was no significant change in the rate of recovery, as compared to that in Period I, it could not be determined whether the observed effects were due to the specific action of these supplements, or to prolongation of the period of bed-rest and alcohol withdrawal. It may not be possible to evaluate the role of dietary supplements in the treatment of cirrhosis until the factors responsible for improvement during the control period are better understood, and until the maximum effects of the basic regime have been determined.

The methods of study employed by Volwiler and his associates (18) were sufficiently similar

to ours to permit some comparisons of the effects of their high protein, high caloric diet with those of the basic diet used in these experiments, under the same conditions of restricted activity in a hospital. There was no significant difference in the clinical improvement observed in the two groups, but the subjects on the better diet showed more complete removal of fat histologically, and greater and more consistent increases in serum albumin. These differences, however, may not have been due to the diet, since Volwiler's subjects were less severely ill than ours, judged both clinically by the incidence of ascites and jaundice, and histologically by the degree of fibrosis present. Only four of his 13 subjects had ascites, compared to 10 of the 14 on the basic diet, and only six showed more than one-plus fibrosis histologically, compared to 13 in our group. On both diets the only significant rises in serum protein occurred in the non-ascitic group, and in several instances the level fell when ascites was present. There was no clear-cut correlation, however, between the degree of fibrosis and the rate of fat removal from the liver. While the differences in the rate of fat removal in the two groups may have been related to differences in the severity and duration of the disease, the possibility that they were due to the diet cannot be excluded.

Franklin and his associates (17) have also studied cirrhotics by the serial biopsy technique, and have concluded that supplements of choline and methionine have specific therapeutic effects. Their results are difficult to interpret since they had no control group, and since their basic diet had a relatively high protein and caloric content and included large supplements of vitamins. Volwiler (18), under somewhat different conditions, on the other hand, has found that lipotropes do not augment the effects of a high protein diet. Franklin's comments are of interest in connection with the observations being reported: "Shorter periods of observation are desirable in view of possible spontaneous remissions which may occur with longer control periods. Even in the shorter periods used in this study, however, the possibility of spontaneous improvement may raise difficulties in evaluating the role of therapy."

Further work is needed to determine what factors were operative during our control period to account for the improvement which occurred.

The possibility cannot be excluded that the basic diet employed was an important factor. The optimal protein requirement for the normal adult is still a matter of speculation, and it is known to be influenced by a great many factors. The standard of 1 gm. per kilogram of body weight daily, however, is widely accepted, and it is generally agreed that larger amounts are required in overcoming malnutrition and in facilitating tissue repair. Accordingly, the requirement in cirrhosis is usually given as 1.75 to 2.0 gm. per kilogram, although it is recognized that these values have not been established under varying dietary conditions (18). By this standard the basic diet was suboptimal in regard to protein. While the caloric supply appeared to be inadequate for some of the subjects, there was no clear-cut evidence that there was a deficiency of protein. Eight of the subjects lost weight on the diet, but in only a few was there obvious tissue wasting, while in the others a decline in weight due to water loss could not be excluded. It is quite possible that under the experimental conditions of bed-rest, a moderate carbohydrate supply and a maintenance caloric intake, 1 gm. of protein per kilogram met both the general nutritional needs and the specific requirements of the liver. The somewhat slower removal of fat from the liver, compared to Volwiler's experience (18), and the fall in serum proteins in some of the subjects are the only indications that the protein intake may have been suboptimal in some instances.

The nutritional requirements of the liver are not known precisely, nor have the factors which influence them been studied in man. In animals, at least, there appears to be a relationship between the requirement of lipotropic substances and the over-all metabolic activity (37). Thus, deficient diets which retard growth (38), starvation (39) and the administration of thiouracil (40), lower the choline requirements of rats. There is suggestive evidence that a similar relationship may exist in man. Low protein diets known to produce fatty infiltration and cirrhosis of the liver in children are usually high in carbohydrate and caloric content (41). Low caloric, low protein diets, on the other hand, do not produce liver damage in man, under some circumstances at least (42). It is conceivable, then, that the decrease in liver fat observed in our experiments, and even the improvement in liver function, were starvation effects.

This might account for the apparent histologic improvement despite obvious clinical deterioration in Case 14, and for the changes observed in the subjects who lost weight during the experiment, but could hardly account for the improvement which occurred in those who gained weight.

If the choline requirement of the liver is related to the caloric intake, alcoholism may play a dual role in creating a choline deficiency—first, by raising the caloric intake without a concomitant increase in protein, and secondly, by reducing the food intake. The caloric value of alcohol is usually disregarded even in well-controlled, paired-feeding experiments (20), and may account for the apparent failure of a normal protein intake to protect the liver against fat infiltration. Moreover, a large enough supplement of choline or protein might prevent or reduce fat infiltration and mask the effects of alcohol, and might account for the failure of some observers (11, 18) to demonstrate any deleterious effects when alcohol is administered to cirrhotics on high-protein diets.

The importance of bed-rest in acute liver disease has been recognized for some time, and recently it has been stressed in the treatment of cirrhosis (18). It is not known whether rest exerts its favorable action by promoting healing, as it does in other inflammatory diseases, or whether it does so by altering the metabolic activities of the liver in some equally obscure way. The decrease in hepatic blood flow during exercise (43) may be of importance in this connection. The degree of physical activity may also be one of the factors which determine the lipotropic requirement of the liver, just as in the case of the rate of growth.

It should be emphasized that whatever the specific effects of the diet on the liver prove to be, the patient with Laennec's cirrhosis will still require a well-balanced diet to overcome the usually associated malnutrition. Future studies may reveal that dietary supplements have no specific effects on the liver, but rather improve the nutrition generally permitting the liver to heal under more favorable conditions.

SUMMARY AND CONCLUSIONS

1. Significant clinical, functional and histological improvement has been observed in patients with Laennec's cirrhosis under controlled condi-

tions of bed-rest, alcohol withdrawal and a minimal basic diet.

2. The effects of such a control period must be taken into account in evaluating the specific effects of dietary supplements on the liver.

3. The results do not warrant any conclusions regarding the relative importance of bed-rest, alcohol withdrawal and diet in producing the effects observed during the control period.

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OXYGEN SATURATION OF STERNAL MARROW BLOOD IN POLYCYTHEMIA VERA¹

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The polycythemia observed at high altitudes and in certain forms of pulmonary and congenital heart disease has led to the acceptance of the view that anoxia is an erythropoietic stimulus. In polycythemia vera, however, the oxygen saturation of the arterial blood has been found to be normal. Observers have, therefore, sought in the bone marrow itself for evidence of anoxia in this disease. Reznikoff *et al.* (1) demonstrated changes in the capillaries and arterioles in the bone marrow of patients with polycythemia vera and postulated decreased blood flow with subsequent anoxia as the erythropoietic stimulus. Chemical evidence of such anoxia has as yet not been obtained.

It has likewise been maintained that marrow anoxia is the stimulus to red cell production in anemia, as for example, after hemorrhage. Recently, however, Grant and Root (2, 3) have determined the percentage saturation and oxygen tension of bone marrow blood in dogs made anemic by bleeding and found no significant difference from the control animals.

These considerations led us to a study of the oxygen content in bone marrow blood in various diseases in man, adapting the method of Grant and Root.

After the studies to be reported in this paper had been completed, the work of Berk, Burchenal, Wood, and Castle (4) on the same subject was published.

MATERIALS AND METHODS

Fifty-two sets of determinations in 50 patients are presented here. From each patient, within a period of several minutes, specimens of bone marrow blood and brachial arterial blood were obtained. In 35 instances antecubital venous blood was drawn without stasis. All bloods were chilled until the analyses were performed.

At the time of withdrawal of the blood specimens for analysis, the patients were recumbent in bed, having

rested quietly for at least ten minutes. Experiments were usually performed between 9 and 10 o'clock in the morning, about two hours after breakfast. More attention was not paid to the establishment of absolutely basal conditions because the act of sternal marrow puncture induced a degree of anxiety in most patients which was of far greater moment than the other factors affecting the basal state.

The sternal bone marrow aspiration was performed by the usual technique at the level of the second interspace with a number 17 bone marrow needle and stylet. After the marrow had been entered, the stylet was withdrawn. In only two patients, one with polycythemia vera and one with an hemangioma of the spleen (not included in the present series), was there a spontaneous welling up of blood into the hub of the needle. In the other patients, a preliminary gentle aspiration with a 10 cc. syringe was performed until blood just appeared in the syringe. The syringe was then discarded and the patients were instructed to perform a momentary Valsalva experiment when necessary, whereupon bone marrow blood was seen to rise into the hub. The tip of a Roughton-Scholander micro-pipette, containing dried heparin, was immediately inserted well below the level of the blood to the junction of the hub with the needle proper. The operator's finger was held over the opposite end in order to prevent blood in contact with air from entering the pipette. Forty cu. mm. were aspirated and transferred to a Roughton-Scholander analyzer for immediate determination of oxygen content. From 0.75 cc. to 1.0 cc. of bone marrow blood was then withdrawn with gentle suction into a 2 cc. syringe containing dried heparin for the determination of oxygen capacity.

Oxygen determinations were performed by the method of Roughton and Scholander (5). For the capacity measurement blood was saturated with oxygen by the aeration of 0.5 to 1.0 cc. for 15 minutes in the syringe in which the specimen had been collected. Correction for dissolved oxygen in both the saturated and unsaturated specimens was made using the figures of Sendroy *et al.* (6).

The patients were divided into four groups, namely, controls, patients with polycythemia vera, anemia and anoxic anoxia. The "control" group consisted of afebrile patients, some of whom were convalescent from a variety of diseases and who manifested no hematologic, cardiovascular, or pulmonary abnormalities. The polycythemia vera group consisted of all patients with this diagnosis, regardless of the stage of therapy. The anemics consisted of patients whose arterial oxygen capacity was less than

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15 volumes per cent, corresponding to a hemoglobin content of 11.0 grams per 100 cc. The anoxic group included patients whose arterial oxygen saturation was below normal. In addition to the anoxic anoxia which all patients in this group manifested, some were presumed to have stagnant anoxia on the basis of congestive heart failure.

In the collection of "bone marrow blood" for the determination of oxygen content and capacity several possible sources of error are to be considered:

1. As indicated previously a preliminary aspiration with a 10 cc. syringe and the performance of the Valsalva maneuver were usually necessary in order to obtain a free flow of bone marrow blood into the hub of the needle. Did these pressures induce changes in the oxygen content of the bone marrow blood obtained? For a brief period the level of bone marrow blood in the hub was exposed to air. Did this brief exposure also induce changes in the oxygen content of the specimen drawn? It is likely that whatever error was introduced affected the controls and the test groups similarly. It was believed that such changes took place only at the surface of the blood column and that errors from these sources could be eliminated by inserting the tip of the pipette well below the level of the blood. This opinion was substantiated by subjecting blood to similar treatment in a dummy experiment.

2. Another source of error is that "bone marrow blood" is not a relatively constant mixture like peripheral blood. When material is aspirated through a needle in the bone marrow, a variable proportion of marrow elements, stagnant red blood cells, and actively flowing blood is obtained. This is evident grossly in that some bone marrow specimens appear homogeneous whereas in others yellowish grey particles are seen. In addition, changes in circulation may have occurred at the tip of the needle in the marrow cavity. An indication of the magnitude of these errors can be obtained by comparing the oxygen capacities of bone marrow and peripheral blood in the same individual. The value, arterial blood oxygen capacity minus bone marrow blood oxygen capacity, had an extreme range from +2.3 to -1.1 volumes per cent. The mean difference, however, was +0.3 volumes per cent, just beyond the error of the method, showing that there was a slight dilution by bone marrow elements. Using the method of Grant (7), in a number of instances, we determined the oxygen capacity of bone marrow blood drawn before and after the removal of 1 cc. and found a similar variation. It should be pointed out in this connection that Grant and Root (2) found an average difference of +1.3 volumes per cent between venous and bone marrow oxygen capacity in dogs. As an indication of the degree of error brought about by variation in the oxygen capacity of bone marrow blood, we have calculated the percentage saturation of bone marrow blood on the basis of the capacity of both bone marrow and arterial blood. The mean and the standard deviation in the control and in the other groups were not changed significantly by either method of calculation. We have therefore used the figures for percentage saturation of bone marrow

blood calculated on the basis of bone marrow capacity in the presentation of our results.

RESULTS

The results in individual cases are shown in Tables I through IV. Table V compares the results in the four groups of cases. The oxygen contents are given in volumes per cent. Letter A refers to brachial arterial blood and letters BM to sternal bone marrow blood.

TABLE I
Control patients

Age, Sex	Oxygen capacity		Oxygen content		Percentage saturation		Difference oxygen content A minus BM
	A	BM	A	BM	A	BM	
62 M	20.3	20.8	20.0	16.3	97	78	3.7
52 M	15.9	15.7	15.3	12.7	94	80	2.6
14 M	19.8	19.5	—	17.1	—	87	—
27 M	16.5	16.1	16.3	11.5	97	71	4.8
69 M	16.6	16.5	15.6	14.3	92	86	1.3
41 M	21.0	20.7	20.4	15.7	96	75	4.7
60 M	18.0	18.6	17.8	15.9	97	85	1.9
21 M	20.3	19.3	19.4	16.0	94	83	3.4
28 M	20.3	20.5	19.9	18.0	97	87	1.9
54 M	17.5	17.1	16.5	11.4	93	66	5.1
49 M	17.2	16.8	16.7	13.2	95	79	3.5
18 M	20.4	20.6	20.6	15.0	99.5	72	5.6
49 M	20.3	20.3	19.6	17.3	95	85	2.3
63 M	19.8	19.5	19.5	16.3	97	83	3.2
49 F	16.5	16.3	16.1	12.6	96	77	3.5
16 M	19.2	18.4	18.7	15.8	96	85	2.9
59 M	16.3	16.4	15.4	13.0	93	79	2.4
27 M	16.8	17.9	17.1	12.6	100	70	4.5
63 M	—	17.6	—	13.4	—	76	—
46 F	16.9	15.7	16.9	10.3	98	65	6.6
60 F	—	16.6	—	12.3	—	74	—
33 M	18.9	19.0	18.3	15.5	95	81	2.8
64 M	18.6	18.4	17.5	15.1	93	82	2.4
55 M	16.6	16.5	16.3	14.2	96	86	2.1
50 M	16.6	17.1	16.3	13.5	96	78	2.8

Percentage saturation of bone marrow blood with oxygen: It is evident that there was a wide variation in the percentage saturation of bone marrow blood with oxygen in all four groups, this being especially pronounced in the control group. The values for patients with anemia tended to fall in the lower range of the control values, but the difference between the means was not statistically significant. The values for the anoxic patients had a similar, but somewhat wider range, and the mean was somewhat lower. The difference between the means of the control and anoxic groups was on the border of significance (p was between

TABLE II
Polycythemia vera patients

Age, Sex	Duration disease	Previous treatment	Hematocrit	Oxygen capacity		Oxygen content		Percentage saturation		Difference oxygen content
				A	BM	A	BM	A	BM	A minus BM
60 F	9	1, 2, 4	57	17.1	16.4	16.9	14.7	97	88	2.2
56 F	1	2, 4	67	23.4	23.3	23.0	19.8	97	85	3.2
77 F	5	2, 4	67	24.2	24.0	23.2	22.0	95	91	1.2
65 M	9	2, 4	61.5	18.6	18.5	18.7	17.7	99	94	1.0
64 M	7	2, 3, 4	62	25.6	24.7	24.8	24.3	96	97	0.5
54 M	12	1, 2, 4	48	17.3	16.8	17.0	13.5	97	80	3.5
55 F*	1	None	70	23.4	22.5	23.2	21.8	98	96	1.4
55 F*	1	None	69	22.3	21.5	21.4	20.5	95	95	0.9
55 F*	1	2	64	21.2	21.3	20.8	17.7	97	83	3.1
56 M	10	4	70	26.3	26.5	25.7	22.7	97	85	3.0
55 M	12	1, 2, 4	60	18.8	19.0	18.0	16.8	94	87	1.2

* Same patient.

Previous treatment:
1. Total body irradiation.
2. Phlebotomies.
3. Phenylhydrazine.
4. P³².

TABLE III
Anemic patients

Age, Sex	Oxygen capacity		Oxygen content		Percentage saturation		Difference oxygen content
	A	BM	A	BM	A	BM	A minus BM
39 M	11.8	12.1	12.0	9.1	99	74	2.9
62 F	12.0	11.6	11.9	9.1	97	78	2.8
60 M	9.9	9.7	9.6	7.8	94	79	1.8
40 F	7.6	7.1	7.7	4.9	97	68	2.8
43 F	5.0	5.3	4.7	4.1	90	75	0.6
56 M	11.0	10.6	10.9	7.1	96	66	3.8
58 M	10.7	11.3	10.3	9.0	94	79	1.3

The first and last cases are pernicious anemia; the others are hypochromic anemia.

0.05 and 0.01).² On the other hand, the values for patients with polycythemia vera were higher than those of the control group, although there was overlapping in the treated polycythemics with relatively low oxygen capacities. The difference between the means was statistically significant (p was less than 0.001).² *A fortiori*, the difference between the means in the polycythemia vera group and the anoxic group was highly significant.

Arterial-bone marrow oxygen difference: The difference between the oxygen content of arterial

² p is the probability that the observed difference between the means is due to chance, as calculated by Fisher's "t" test.

TABLE IV
Anoxic patients

Diagnosis	Age, Sex	Oxygen capacity		Oxygen content		Percentage saturation		Difference oxygen content
		A	BM	A	BM	A	BM	A minus BM
Pulmonary fibrosis	52 M	17.9	—	16.6	12.0	92	—	4.6
Emphysema	62 M	21.7	22.1	17.9	14.0	83	63	3.9
Congestive heart failure	63 M	17.1	15.8	14.4	10.1	84	70	4.3
Pulmonary fibrosis	60 M	21.7	21.2	18.7	15.1	85	71	3.6
Congestive heart failure	60 F	21.0	21.1	19.1	17.3	90	82	1.8
Cor pulmonale	54 M	23.5	23.0	17.5	17.1	74	74	0.4
Tetralogy of Fallot	30 F	25.1	25.6	22.6	18.6	89	72	4.0
Pulmonary fibrosis	42 M	21.5	20.9	19.9	16.4	92	78	3.5

and bone marrow blood (A-BM difference) displayed a similar wide range of variation, greatest in the control and anoxic groups. The A-BM values for the anemics tended to fall in the lower range of normal, but the difference between the means was not significant. The patients with anoxia, due at least in part to the heterogeneous make-up of the group, showed no significant difference from the controls. The A-BM values for patients with polycythemia vera, however, were in general lower than the controls, although there was overlapping. The difference between the means in the latter two groups was statistically significant (p was between 0.01 and 0.001).²

The values for antecubital venous blood oxygen content displayed the wide range of variation previously reported by others. With few exceptions, the venous oxygen content was lower than the bone marrow content.

TABLE V
Mean values for all patients

	Percentage saturation bone marrow blood			Difference oxygen content A minus BM		
	No.	Mean	S.D.	No.	Mean	S.D.
Control	25	78.8	6.44	22	3.35	1.347
Polycythemia vera	12	88.3	5.52	11	1.93	1.094
Anemia	7	74.1	5.27	7	2.29	1.099
Anoxia	7	72.9	6.07	8	3.31	1.489

S.D.—Standard deviation.

DISCUSSION

The wide range of variation in the oxygen content and percentage saturation of bone marrow blood found in this study confirms the results of Grant and Root (2, 3) in dogs and of Berk and co-workers (4) in man.

By the present methods, with their limitations as discussed above, it was not possible to demonstrate bone marrow anoxia in polycythemia vera. In this respect, our findings are in agreement with those of Berk *et al.* However, whereas they found no significant difference in the mean value of the percentage saturation of bone marrow blood in the controls and in polycythemia vera, our findings show a significantly higher value for the latter. In common with Grant and Root (2), we confined our analyses of bone marrow blood oxygen content to

the first several drops of blood appearing in the needle. Berk *et al.* withdrew 5 cc. by syringe in their "old" technique and 1 cc. in their "new" technique performing analyses upon this blood. It is significant that our average value for percentage saturation in the controls, 78.7 per cent, was essentially the same as that obtained by Grant and Root in control dogs. The corresponding value for normals found by Berk *et al.* was 88.6 per cent by their "new" method, which is not significantly different from their value for polycythemias, 87.3 per cent, nor from our value for polycythemia vera, 88.6 per cent. If difference in technique accounts for the varying findings, it is not clear in the comparison of these results why this difference affected only the normals, where we differed by 9.9 per cent, while identical figures were obtained in polycythemia vera. A possible explanation may be that in polycythemia vera the bone marrow is hyperplastic and extraordinarily vascular so that the first cubic centimeter of blood brought into the syringe may represent bone marrow blood. On the other hand, in normal patients, where the bone marrow is not hyperplastic nor unduly vascular, only a portion of this cubic centimeter may come directly from the marrow. The remainder may be drawn in from extra-marrow sources. In support of this explanation is the fact that in four controls, we have found the oxygen content of bone marrow blood drawn after the removal of 1 cc. to be higher than the oxygen content of blood obtained before such removal. This finding, however, is at variance with the results obtained by Grant and Root in dogs, where successive bone marrow specimens showed the same oxygen content.

The other difference in technique, namely, that Berk *et al.* secured a spontaneous flow of marrow blood by having their patients turn to one side whereas we induced flow by gentle suction, does not, in our opinion, account for the different results because of the dummy experiments performed by both sets of investigators, which showed no change as a result of such manipulations.

The percentage saturation of bone marrow blood in polycythemia vera would be higher than normal even if the arterial-bone marrow oxygen difference were the same in the two groups. Actually, the A-BM difference was lower in the polycythemic

group, thus accentuating the difference in percentage saturation. Ordinarily decreased arterio-venous oxygen difference implies increased blood flow, provided oxygen consumption is not decreased. Overall oxygen consumption in polycythemia vera is increased or normal. We are aware of no *in vivo* studies of oxygen consumption of bone marrow itself. One would expect an increased oxygen consumption in hyperplastic marrow. Therefore, increased blood flow compatible with an hyperplastic, highly vascular marrow may be present. This would impart to the circulation in the marrow in polycythemia vera a unique character since there is evidence that the general circulation in this disease is slowed. The theoretical possibility remains, however, that oxygen consumption of bone marrow in polycythemia vera is decreased (histotoxic type of anoxia or arterio-venous shunting). In this case the low A-BM difference would not necessarily indicate increased blood flow.

In agreement with the findings of Grant and Root in post-hemorrhagic anemia in dogs we have been unable to demonstrate a significant difference from the controls in the percentage saturation or arterial-bone marrow oxygen difference of a small number of cases of pernicious and hypochromic anemia. This may be due, in part, to the fact that our methods are not sensitive enough to detect small differences in the face of great normal variation.

In patients with anoxic anoxia the method was adequate to detect low percentage saturation values in the bone marrow blood. That they were not lower is due possibly to various compensatory factors, such as increased blood flow and increased oxygen capacity.

SUMMARY AND CONCLUSIONS

1. Oxygen content, capacity, and percentage saturation of bone marrow blood were determined in man in a group of controls and in patients with polycythemia vera, anemia, and anoxic anoxia. The limitations of the method are discussed.

2. The percentage saturation of bone marrow blood was greater and the arterial-bone marrow blood oxygen difference was smaller in patients with polycythemia vera than in controls. This finding does not support the theory that there is a local bone marrow anoxia due to decreased blood flow in polycythemia vera, but is compatible with either increased blood flow or decreased oxygen utilization by the marrow in this disease.

3. No significant differences were found between anemias and controls, in a small number of cases.

4. In patients with anoxic anoxia the percentage saturation of bone marrow blood with oxygen was lower than in the controls.

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IV. RESPONSE OF CITRIC ACID LEVELS OF NORMAL ADULTS AND CHILDREN TO INTRAMUSCULAR INJECTION OF EPINEPHRINE¹

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Our earlier observations that oral administration of glucose would cause a lowering of citric acid levels in humans (1, 2) led us to investigate the effect of epinephrine on citric acid levels.

One-half minim of a 0.1% solution of epinephrine in water per kilogram of body weight was administered with a maximum dose of ten minims to any individual, after a 12-hour fasting period. Thus all adults and most children studied received a dose of 10 minims intramuscularly. Serum glucose and citric acid levels were followed at short intervals as shown in the charts below. In view of the varying response of different individuals, average curves were not drawn but representative results are shown in Figure 1 for children, and Figure 2 for adults. Glucose determinations were carried out by the method of Folin and Wu (3) and citric acid determinations were carried out as described before (4). This latter method has been in routine use in our laboratory for more than a year and is in error by less than 5%.

Comparison of fasting serum levels of citric acid with the maximum level reached after epinephrine administration is tabulated in Table I. Results obtained on the serum of children and adults are averaged together since no significant difference was noted between the two groups.

TABLE I

Comparison of fasting serum levels of citric acid with citric acid levels obtained after epinephrine administration

No. cases	Average fasting level	Average maximum level	% increase over fasting level	P value fasting vs. max. level
28	mg. % 2.16	mg. % 2.85	32	5×10^{-3}

In order to compare these results with the effect of insulin, similar studies were carried out with insulin administered intramuscularly. This type of study had been reported before but no curves

were shown (1). Figure 3 shows four typical curves obtained in this study.

We have also studied the effect, on citric acid levels, of intravenous administration of glucose (5). Figure 4 shows a typical result obtained after the intravenous administration of glucose to a 100 lb. girl (50 mg./lb.). A total of 5 grams of glucose dissolved in 25 ml. of water was administered within a one-minute period. This curve is included for comparison with the curves obtained on intramuscular administration of epinephrine.

DISCUSSION

It can be seen readily from Figures 1 and 2, and Table I that intramuscular injection of epinephrine will cause a rise in citric acid serum levels. This is in contradistinction to the effect of insulin (Figure 3) or glucose, either orally (1, 2) or intravenously (Figure 4), all of which cause a lowering of citric acid levels.

Earlier (2) we reported that certain convulsives and diabetics responded to administration of glucose with a rise in citric acid levels rather than a fall. We are investigating the possibility that in these cases some abnormality exists which results in excessive epinephrine secretion during the test.

A preliminary drop in citric acid levels, from the fasting level, was observed in 17 out of the 28 cases. The average citric acid level for all cases, 15 minutes after the administration of epinephrine, was 3% lower than the fasting level. Statistical comparison of this level with the fasting level yielded a *P* value of 0.6 which is not statistically significant. Thus it can be said that no significant rise in citric acid levels occurs within the first 15 minutes. The rise in glucose levels during this time (within 15 minutes) is definitely significant, being 50% higher than the fasting level. Compar-

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RESPONSE OF CITRIC ACID AND GLUCOSE LEVELS
TO INTRAMUSCULAR ADMINISTRATION OF EPINEPHRINE (NORMAL CHILDREN)

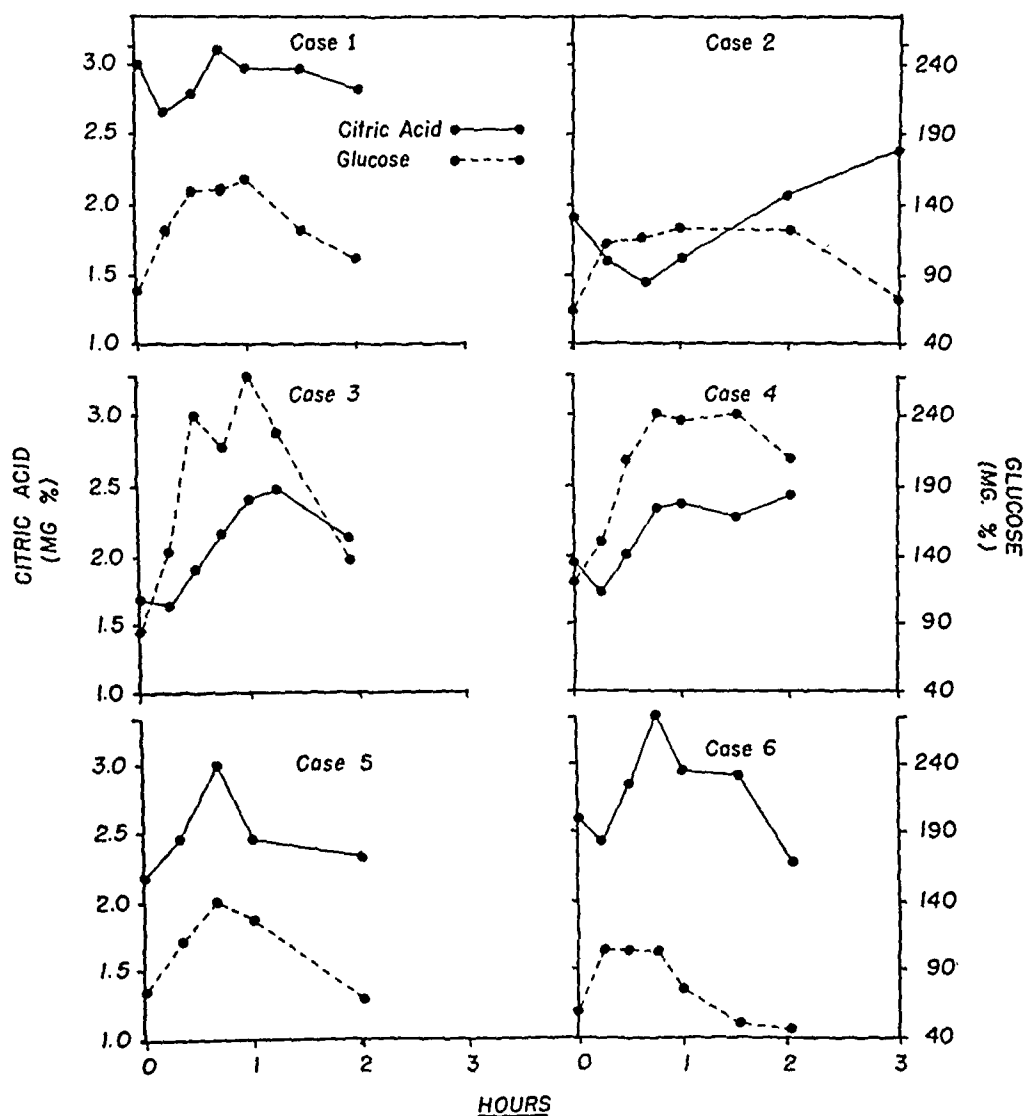


FIG. 1. REPRESENTATIVE CURVES ILLUSTRATING THE EFFECT OF INTRAMUSCULAR INJECTION OF 0.5 MINIMS/KILO OF EPINEPHRINE ON SERUM CITRIC ACID AND GLUCOSE LEVELS (NORMAL CHILDREN)

ing this level with the fasting level a P value of 10^{-7} is obtained. Thus the response in changes of glucose levels on administration of epinephrine is more rapid than the response of citric acid levels. These observations would imply that the action of epinephrine, in its effect on citric acid levels, is probably more indirect than the mechanism whereby it acts in causing a rise in serum glucose levels.

Other acids like pyruvic and lactic acids which are intermediates in glucose metabolism increase when glucose, insulin or epinephrine is administered (6-12). However, the literature is not in

complete agreement as to the effects of insulin and glucose administration on serum lactic acid levels (13).

Citric acid is distinctive in showing a lowering of the serum levels on the administration of insulin or glucose but a rise on administration of epinephrine.

The observation that epinephrine will cause a rise in citric acid levels must be taken into account when explaining changes in citric acid levels under certain abnormal conditions. For example, Lövgren claims that high citric acid levels are found consistently in acute allergic states (14).

This may be associated with an increase in epinephrine secretion observed in this condition (15):

Other substances have been reported as causing increased citric acid levels in the blood. Alwall (16) reports an increase in citric acid levels with prostigmine in man. However, Martensson (17) disagrees with these findings and reports no change. Differences of this type which appear in the literature may be due, in part, to variations in the alarm response of patients under the varying conditions under which the experiments were carried out.

Physostigmine, a compound related in physiological action to prostigmine, is reported to interfere with a rise in citric acid levels which normally follows injection of calcium lactate (18). The action of this drug and others like it which lower citric acid levels should be studied in their rela-

tionship to their anti-epinephrine action and their effect on epinephrine secretion.

Of interest is the observation of Hajdu and Korenyi (19) that continuous injection of citric acid will cause hypertrophy of the medulla of the suprarenals. If the left celiac ganglion is extirpated hypertrophy does not occur. The greatest concentration of citric acid in the body occurs in the suprarenals (20-22).

Synthalin, a guanidine derivative, causes a lowering of glucose levels in diabetics. This compound and guanidine itself will produce a rise in citric acid levels when injected (23). In view of our observations that insulin causes a lowering of citric acid levels, it is apparent that synthalin, as far as its effect on citric acid levels is concerned, is quite different from insulin. It is well known that synthalin produces liver damage (24-26). In

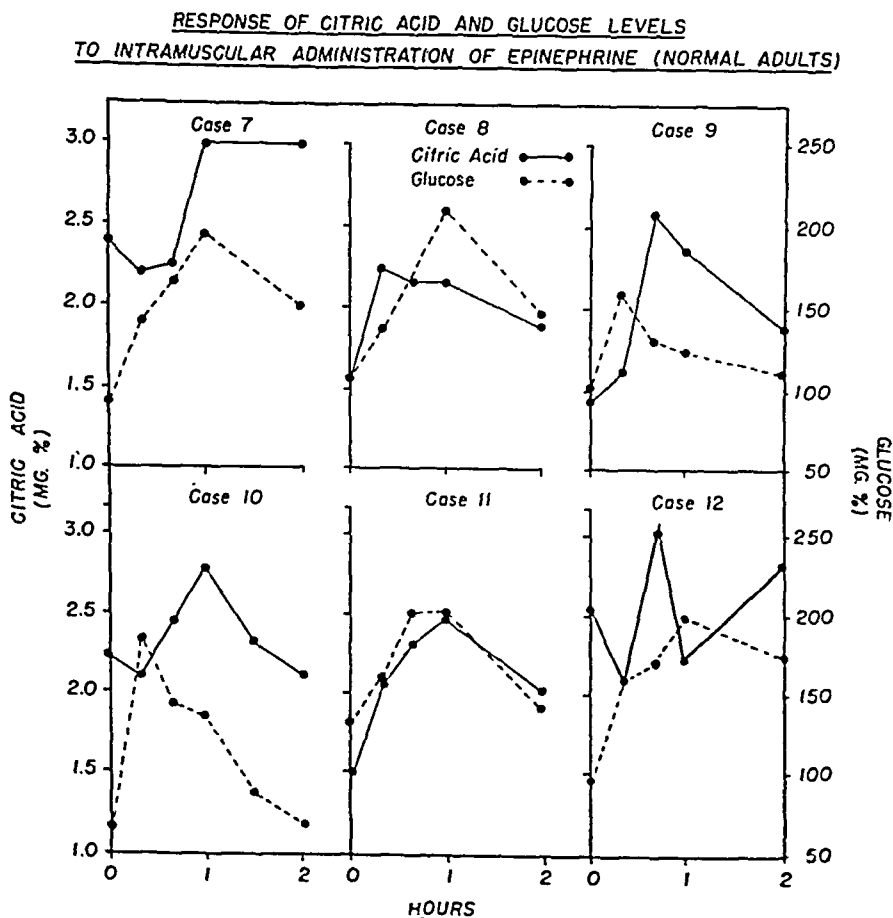


FIG. 2. REPRESENTATIVE CURVES ILLUSTRATING THE EFFECT OF INTRAMUSCULAR INJECTION OF 0.5 MINIMS, KILo OF EPINEPHRINE ON SERUM CITRIC ACID AND GLUCOSE LEVELS (NORMAL ADULTS)

RESPONSE OF CITRIC ACID AND GLUCOSE LEVELS
TO INTRAMUSCULAR ADMINISTRATION OF INSULIN (NORMAL ADULTS)

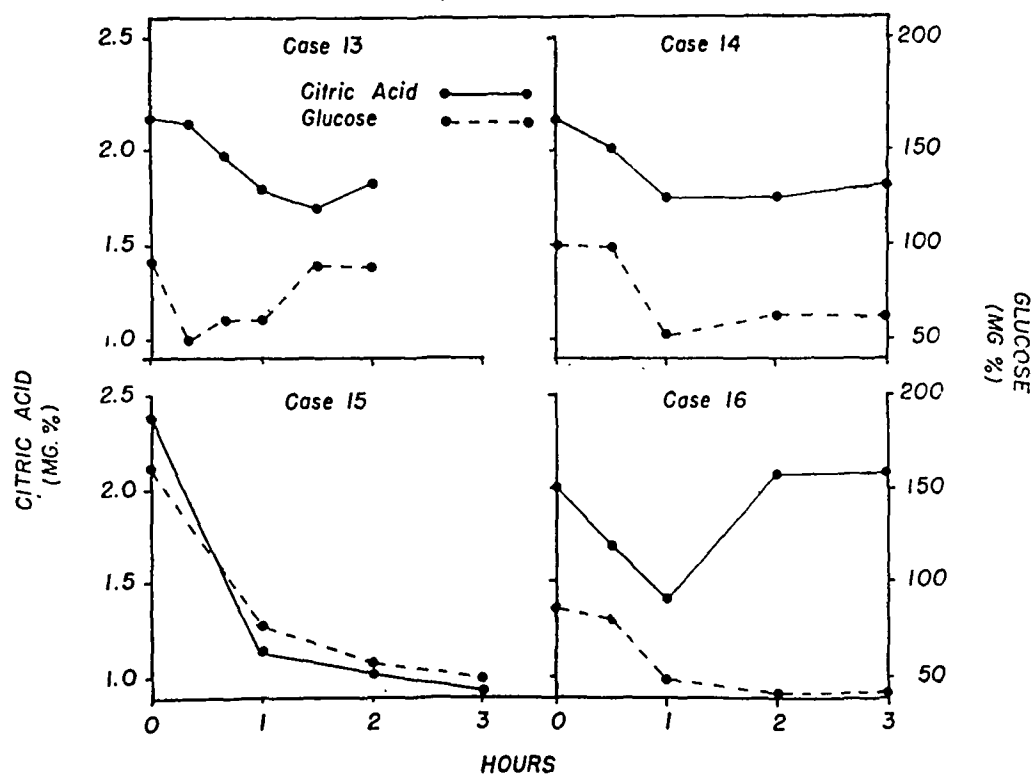


FIG. 3. REPRESENTATIVE CURVES ILLUSTRATING THE EFFECT OF INTRAMUSCULAR INJECTION OF INSULIN ($\frac{1}{2}$ UNIT/KILO) ON SERUM CITRIC AND GLUCOSE LEVELS

RESPONSE OF CITRIC ACID AND GLUCOSE LEVELS
TO INTRAVENOUS ADMINISTRATION
OF GLUCOSE

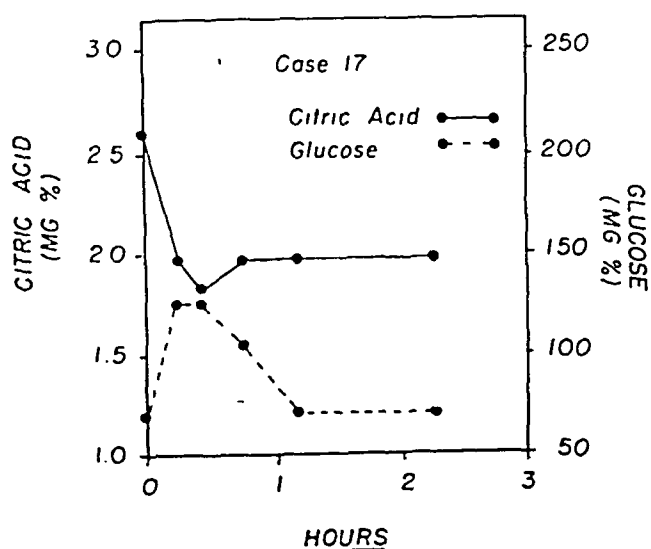


FIG. 4. CHANGING SERUM CITRIC ACID AND GLUCOSE LEVELS AFTER INTRAMUSCULAR INJECTION OF 50 MG./LB. OF GLUCOSE

severe liver damage elevated citric acid levels have been reported (27).

The exact relationship between citric acid and endogenous epinephrine levels will be ascertained only when a precise and specific method for the determination of minute amounts of epinephrine becomes available.

SUMMARY

Epinephrine administered intramuscularly caused a rise in citric acid serum levels in 28 normal adults and children.

Administration of glucose intravenously or insulin intramuscularly results in a decrease in citric acid serum levels.

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STUDIES OF THE EFFECTS OF FLAVONOIDS ON ROENTGEN IRRADIATION DISEASE. II. COMPARISON OF THE PROTECTIVE INFLUENCE OF SOME FLAVONOIDS AND VITAMIN C IN DOGS¹

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The nature of dietary factors contributing to the maintenance of vascular integrity has not, as yet, been satisfactorily elucidated. However, vitamin C appears to play a specific role (1-3) by functioning in some manner to incorporate the vascular cement substance. The significance of other agents in alleviating "vascular purpura" has remained inconclusive.

It has been claimed that "vascular permeability" is dependent upon regulation by the entire class of "vitamin P" substances. Since 1936, the experimental device of eliminating "vitamin P" from the diet of guinea pigs has been the customary approach with markedly contradictory observations recorded (4-10). Recent investigations in this laboratory have utilized a somewhat different principle in a study of exogenous agents contributing to vascular repair (11, 12). Roentgen irradiation disease in dogs induces a train of pathological phenomena secondary to a generalized cellular disturbance (13). Since a purpura has been an invariable feature of controlled roentgen irradiation, anti-hemorrhagic agents have been considered, but vitamins K, C and the B complex and calcium have proven ineffective (14). When the flavonol glucoside, rutin, was given to 350 r whole-body, single-dose irradiated dogs, not only was the hemorrhagic diathesis significantly reduced, but mortality fell from 60 per cent to 11 per cent (11, 12). The control of purpura was achieved in the presence of marked thrombocytopenia and other typical manifestations of irradiation disease.

Thus, it has been proposed that rutin either decreases the rate of vascular disintegration, or increases its repair (12).

The purpose of this report is to summarize briefly observations on the activity of other substances of the flavonoid and related groups and to indicate the relationship of these substances with vitamin C in the irradiated dog.

METHODS

Dogs of the beagle strain, identical to those utilized in the previous study (12) were used throughout. Diet, the manner of animal care and recording of observations were performed as before. Irradiation and hematological methods have already been described (12). The dosage in all these studies delivered to the dorsum of the skin of the dogs was 350 r. This value was established with each irradiation by a direct trial measurement with the Victoreen ionization chamber.

The most reliable and consistent basis for evaluation of the value of anti-irradiation agents has been a consideration of the end-mortality of treated and control dogs. A statistical analysis with the X^2 -test indicated the minimum number of dogs required in the testing of an agent to validate a deviation from control mortality. Further, this analysis assisted to establish the significance within a 2-5 per cent probability level. Table I serves to illustrate this consideration. Thus, based on a mortality of 60 per cent amongst 37 untreated control dogs, an experimental mortality of 10 per cent would have a 74 per cent chance of detection and validation when 10 dogs were used in the trial; 53 per cent when six dogs were used. A mortality of 20 per cent would have a 38 per cent chance of detection when 10 dogs were tested, 26 per cent when using six dogs.

In view of the limited supply of test animals and of some scarce compounds, usually five or six dogs were utilized in each trial. Thus, with numbers such as these, significance can only be attached to results indicating a mortality no greater than one dog in each group of five.

Test substances were usually given in single doses of 50 mg. for purposes of direct comparison with results obtained with the same dose of rutin. The compounds were given orally in gelatin capsules three times daily beginning one week prior to irradiation and continuing until 28 days post-radiation. The consumption of these

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TABLE I

Evaluation of significance of test groups

Probability of detecting a reduction of mortality from 60 per cent (based on a sample of 37 control animals) when the χ^2 -test is used for a test of significance.*

If the number of experimental animals is:	If the significance level for rejection of the null hypothesis is:											
	10-5%				5-2%				2-0%			
	If the true mortality of experimental animals is:											
	10%	20%	30%	40%	10%	20%	30%	40%	10%	20%	30%	40%
6	53	26	12	5	53	26	12	5	0	0	0	0
8	81	50	26	11	81	50	26	11	43	17	6	2
10	93	68	38	17	74	38	15	5	74	38	15	5
12	97	79	49	22	89	56	25	8	66	27	8	2
15	99	84	51	22	94	65	30	9	82	40	13	3
20	100	91	61	25	99	80	42	13	96	63	24	5

* Certain minor inconsistencies result from approximations in the application of χ^2 .

test substances was facilitated by incorporating the capsule in approximately 10-20 grams of fresh ground beef. The control dogs received an equal quantity of meat three times daily.

EXPERIMENTAL

Biologic response to irradiation

When 37 dogs were given the standard total body single dose of 350 r roentgen irradiation, typical leukopenia, thrombocytopenia and anemia were followed by hemorrhagic signs and gross bleeding in 64 per cent, while 60 per cent of the dogs succumbed in 20 (13-30) days. When the flavonol glucoside, rutin, was administered continually pre- and post-radiation to 27 dogs, 22 per cent developed a purpuric tendency and only 11 per cent died in 21 (16-31) days (Table II). A significant change from untreated controls was a reduction in the hemorrhagic diathesis (11, 12). It has already been noted that rutin had little or no effect upon the panhematopenia of the irradiation disease.

Effect of flavonoids in irradiated dogs

A variety of substances in the flavonoid series have been tested in the standard manner in the 350 r irradiated dog. These include the flavonol glucosides hesperidin,² naringin² and quercitrin,⁴

²Furnished by Mr. A. J. Lorenz, Director of the laboratories, and Mr. W. E. Baier, Manager, California Fruit Growers' Exchange, Ontario, California.

⁴Obtained from S. B. Penick & Co., New York, N. Y.

TABLE II

"Active" flavonoids influencing the course of irradiation disease

	Number of dogs		Mortality	Survival post-radiation		Exhibiting gross bleeding*
	Started	Survived		Average days	Range days	
Control	37	15	60	20	13-30	64
Rutin	27	24	11	21	16-31	22
Hesperidin	6	5	17	19	—	100
Morin	6	6	0	0	—	0
Homoeriodictyol	5	5	0	0	—	20
Epim-d-catechin	10	9	10	15	—	50

* This includes even mild gingival oozing and petechiae formation.

the aglucone, quercetin,⁵ and the derivatives, epimerized d-catechin,⁶ homoeriodictyol,⁴ hesperidin methyl chalcone,² esculin⁷ and morin.⁸ Because of the distinct differences in protective effectiveness observed with these substances it has been convenient to classify them roughly as "active" and "inactive." Accordingly, the results of these tests are summarized in Tables II and III. Data

⁵Furnished by Dr. J. F. Couch, Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pennsylvania.

⁶Furnished through Dr. E. L. Sevringhaus, Hoffmann-La Roche, Inc., Nutley, New Jersey.

⁷Obtained from the Mercantile Import Company, New York, N. Y.

⁸Obtained from the Eastman Kodak Company, Rochester, New York.

TABLE III

Substances with no activity in influencing the course of irradiation disease

	Number of dogs		Mortality	Post-radiation survival		Exhibiting gross bleeding
	Started	Survived	per cent	Average days	Range days	per cent
Control	37	15	60	20	13-20	64
Hesperidin methyl chalcone	6	2	67	17	13-22	100
Quercitin	6	3	50	14	12-15	67
Esculin	6	3	50	15	13-19	100
Quercitrin	9	5	44	16	12-22	78
Naringin	6	3	50	21	17-28	50
Sodium gentisate	5	2	40	19	15-22	80
Dopa	6	3	50	14	12-15	50

obtained with the feeding of rutin are given in Table II to provide some basis of comparison.

In brief, it is to be noted that as observed with rutin, treatment with hesperidin, epimerized d-catechin, homoeriodictyol and morin reduced the incidence of hemorrhage and mortality of the irradiated dogs (Table II). Animals which succumbed from the effects of irradiation were grossly similar whether or not a flavonoid was given; hypoplasia of hematopoietic centers, generalized purpura and visceral hemorrhages were always observed. In general, dogs which survived exhibited fewer and smaller induced petechiae. The results from careful hematological studies can be summarized as follows: averaged values from dogs treated with the substances listed in Table III were identical with those of the untreated dogs while the changes seen in the peripheral blood of dogs given the compounds in Table II resembled those recorded for rutin-treated irradiated dogs (12). In the latter, the significant differences observed were a reduced rate of anemia formation, an increase in erythroblasts and nucleated red blood cells, and a significantly lower sedimentation rate.

Effect of other agents in irradiated dogs

Since flavanones can be degraded *in vitro* to a benzopyrone, it appeared possible that the latter type of compound might retain certain of the anti-irradiation effects of the parent flavonoids. Accordingly, coumarin⁸ was selected as an illustrative compound and tested in the usual fashion. Of six dogs, only two (33 per cent) succumbed 15

(12-19) days after irradiation with some reduction in hemorrhagic tendencies.

In vitro tests (15) have indicated that members of the "vitamin P" group are effective in inhibiting the activity of hyaluronidase. A suggestion was entertained that increased hyaluronidase activity in the roentgen irradiation disease might induce certain features of the vascular dysfunction and "disintegration" (16).⁹ This was fortified by reports that "vitamin P" compounds possessed anti-hyaluronidase activity in *in vitro* tests (15). The recent note of Meyer (17) of potent hyaluronidase inhibitors in the quinoid structures raised the possibility that these substances might also influence the course of irradiation disease. Thus, 3, 4-dihydroxyphenyl alanine (dopa)⁶ and sodium gentisate⁶ were tested in the standard manner and the data are summarized in Table III. Although only two of five dogs (40 per cent) treated with sodium gentisate succumbed, the surviving animals were critically ill. It is to be noted that in so small a test series, the fate of one animal will influence the mortality by 20 per cent. In no significant manner did the hyaluronidase inhibitors influence the course of irradiation disease in dogs.

Effect of ascorbic acid on the anti-irradiation activity of vitamin P compounds

Twelve dogs were given 100 mg. of 1-ascorbic acid orally three times daily beginning one week pre-radiation, and after the standard exposure to 350 r the test substance was given four additional weeks (12). Six dogs (50 per cent) succumbed after 12-14 days post-radiation and the clinical course and hematological data of all animals were identical to the control irradiated dogs (Table IV). Subsequently this treatment, which had been ineffective in itself, was combined with the substances selected from the "inactive" list in Table III. These were hesperidin methyl chalcone and quercitin. Thus, 100 mg. of ascorbic acid plus 50 mg. of hesperidin methyl chalcone were given orally three times daily to five dogs, and 100 mg. of ascorbic acid plus 50 mg. of quercitin were given to 10 dogs. The medication was continuous through-

⁹ In other studies, we have observed that neither irradiation disease nor rutin nor a combination of both, produced an alteration in the urinary excretion of hyaluronidase from the dog. Incubating dog blood with hyaluronidase resulted in no hematological changes.

TABLE IV

Effect of supplementing ascorbic acid with inactive flavonoids on the course of irradiation disease

	Number of dogs		Mortality	Survival post-radiation		Exhibiting gross bleeding
	Started	Survived	per cent	Average days	Range days	per cent
Control	37	15	60	20	13-20	64
Ascorbic acid*	12	6	50	13	12-14	100
Ascorbic acid plus quercetin	10	9	10	11	—	70
Ascorbic acid plus hesperidin methyl chalcone	5	1	80	19	17-21	80

* 100 mg. by mouth given three times daily beginning seven days before irradiation and thereafter for 28 days.

out in the usual manner from one week pre-radiation to four weeks post-radiation. The results of these tests are summarized in Table IV. The hematological data from all groups listed in this table were very similar.

DISCUSSION

It has been suggested that the inconsistencies in the past studies on "vitamin P" have arisen primarily because the need for this agent is not easily demonstrated under normal physiologic circumstances. Another complication encountered has been the derivation of an adequate diet free of the universally distributed flavonoids. A further deterrent has been an ignorance of the species requirements for "vitamin P." Certainly, the latter has been a formidable obstacle in the program of this laboratory and to date, consistent data have been obtained only with the dog but not with the rabbit, rat, guinea pig or mouse.¹⁰

The technique of acute whole-body, mid-lethal irradiation in the dog produces predominantly a vascular derangement detectable most directly in the clinical course of the animal. Autopsy has demonstrated the existence of multiple widespread vascular lesions although the usual histopathological examination of vessels in the irradiated tissues has not revealed the origin of the disintegration. The nature of a true "vascular purpura" is discussed by Hiramatsu (18). He differentiates this

state from a "purpura of the blood" which includes a thrombocytopenia and presumably the existence of any anticoagulant such as heparin (14).

In the course of these studies with the flavanones it has been observed that neither is the course of the usual post-radiation panhematopenia and severe thrombopenia altered significantly by protective flavonoids, nor has it been observed that these agents inhibit heparin.¹¹ Thus, it might appear that these agents affect the vascular system directly, perhaps participating as a principal in the "wear and tear" of a part or all of the vascular system, inhibiting its degeneration and either stimulating, or being incorporated in, its regeneration. In support of this proposition is the observation that the process of vascular disintegration together with universal cellular catabolism and necrosis are accelerated in the irradiated animal. It appears that the "strain" of repair elicits the positive anti-hemorrhagic effects of the flavonoids in the mid-lethal irradiated dog when the usual dietary complement of these agents becomes inadequate.

"Vitamin P" has been identified as a wide variety of flavonoid compounds in the past (4, 5, 19-21). Claims of activity have been made for many of the substances included in the present report. Eventually our present program envisions the "screening" of an even larger number of compounds with the dog irradiation technique and the results of these tests will be given on subsequent occasions. However, it appears that the following flavanones possess in common, an approximately equal capacity to protect the 350 r x-irradiated dog; they are rutin, hesperidin, epimerized d-catechin, homoeriodictyol and morin. It is quite possible that there exists a considerable qualitative and quantitative variation of anti-hemorrhagic capacity amongst these substances. However, it would be hazardous to draw anything but limited conclusions from the present data. The purpose of this report is simply to identify compounds which have "protected" the irradiated dog under given specific conditions.

Since the "active" agents possess the same fundamental flavanone structure it appeared incon-

¹⁰ Field, J. B., and Rekers, P. E. To be published.

¹¹ Heparin will increase the sedimentation rate of blood, and although rutin *in vivo* and *in vitro* reduces increased sedimentation rates, it does not affect the prolonged coagulation time of heparinized blood.

sistent that other very similar compounds and flavonol glucosides as quercitrin and naringin failed to influence the course of irradiated dogs. Particularly disappointing was the performance of quercitin, the aglucone of rutin. With the latter substance only 11 per cent of the dogs succumbed to the effects of irradiation. Given quercitin, 50 per cent of the dogs died with severe irradiation disease. Thus, the demonstration of the synergistic action of ascorbic acid when given concomitantly with quercitin, which reduced mortality to 10 per cent, is of considerable interest. The inter-relationship of vitamin C and "vitamin P" has been sharply debated. Zilva (8) indicated that ascorbic acid alone would maintain capillary repair while Szent-Gyorgyi suggested that ascorbic acid probably exerts a catalytic effect potentiating the action of "vitamin P." The present data support the latter view and it may be predicted that supplementary treatment with ascorbic acid may potentiate an anti-hemorrhagic action of other flavanones as naringin and quercitrin reported at present as "inactive." Study of this problem is in progress.

Apparently degradation of the parent flavanone molecule sacrifices anti-hemorrhagic activity. Thus, esculin and hesperidin methyl chalcone failed to "protect" irradiated dogs even when ascorbic acid supplemented the chalcone in one test. These findings are in disagreement with reports of high "vitamin P" activity for these substances as derived through indirect assay methods (20, 21), or when petechiae formation was measured in rats with a thrombocytopenia resulting from treatment with an anti-platelet serum (22). Two of the six dogs (33 per cent) given coumarin succumbed compared with a mortality of 60 per cent in untreated dogs. Until a larger series of dogs is tested, this result cannot be interpreted as any more than a suggestion that a molecule smaller than the flavanones may "protect" irradiated dogs.

The possibility that the "spreading factor," hyaluronidase, plays a role in the course of roentgen irradiation disease and may be implicated "in accentuating capillary fragility rather than inducing direct change in capillary permeability" (16) appears unlikely. This impression is supported by the failure of potent hyaluronidase inhibitors as dopa and sodium gentisate to influence the course of irradiation disease. Contrary to other

claims (15) that "vitamin P" inhibits hyaluronidase, neither has the administration of large quantities of flavonoids had any effect on the hyaluronidase levels in the urine of the normal dog nor have preliminary tests implicated hyaluronidase in the evolution of roentgen irradiation disease of dogs.

SUMMARY

1. Exposed to 350 r single-dose, total-body x-irradiation, 60 per cent (22 of 37) untreated control dogs succumbed with a prominent hemorrhagic syndrome. Five flavanone substances appeared roughly of equal activity in reducing the hemorrhagic signs of the irradiation disease when administered continuously pre- and post-radiation. When rutin was fed, 11 per cent (three of 27) of the dogs succumbed; with hesperidin, 17 per cent (one of six); with epimerized d-catechin, 10 per cent (one of 10); and with homoeriodictyol none of five and with morin, none of six irradiated dogs succumbed.

2. Other flavonoids and their derivatives did not significantly reduce either the mortality or hemorrhagic changes in irradiated dogs. These included hesperidin methyl chalcone, esculin, quercitrin, quercitrin and naringin. With the benzopyrone, coumarin, 33 per cent (two of six) of the dogs succumbed suggesting slight anti-irradiation activity.

3. Ascorbic acid alone failed to influence the course of irradiation disease and 50 per cent (six of 12) of treated dogs died. However, when ascorbic acid was given simultaneously with quercitrin which by itself was ineffective (50 per cent mortality), only 10 per cent (one of 10) dogs succumbed exhibiting reduced signs of the disease. No increase in protective activity occurred when ascorbic acid and hesperidin methyl chalcone were given together.

4. It is suggested that previous misunderstanding of the nature of "vitamin P" has arisen from both the failure to recognize that several flavanone analogues possess very similar anti-hemorrhagic "activity" and that ascorbic acid has the capacity to potentiate "activity" in other flavanones.

5. Potent hyaluronidase inhibitors as dopa and sodium gentisate failed to influence the course of irradiation disease in dogs. It is suggested that the "spreading factor," hyaluronidase, plays little or no role in the evolution of this disorder.

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THE PLASMA VISCOSITY IN PULMONARY TUBERCULOSIS AND RHEUMATIC DISEASES

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INTRODUCTION

In 1940 T'ang and Wang (1) measured the erythrocyte sedimentation rate and the plasma viscosity in 176 cases of pulmonary tuberculosis and 56 cases of tuberculosis of other organs. These writers concluded from their data that the plasma viscosity determination may be a more sensitive test for activity of tuberculosis than the sedimentation rate.

Independently, in 1942, Whittington (2) in a study of the mechanics of the erythrocyte sedimentation rate suggested that this procedure might with advantage be replaced by plasma viscosity measurements.

The method was tried on 63 tuberculous patients by Miller and Whittington (3) who found that the plasma viscosity was a more reliable index of the clinical condition than the erythrocyte sedimentation rate. The general findings of the latter paper were confirmed by Houston, Harkness and Whittington (4) and by Harkness, Houston and Whittington (5) after 645 estimations of the plasma viscosity—223 of these were of pulmonary tuberculosis; 385 were of a variety of diseases and 37 were of normal persons. Although these data indicate that the plasma viscosity is significant in following the course of persons with pulmonary tuberculosis, they show emphatically that the test is non-specific.

For the past 20 years the accepted laboratory method of estimating the general reaction of rheumatic diseases has been the erythrocyte sedimentation rate. From this earlier experience, a trial of the plasma viscosity test in rheumatic diseases also seemed desirable. Cowan and Harkness (6) have reported 320 viscosity estimations made at the Royal Infirmary, Sunderland, in various rheumatic diseases. They found that the viscosity test was much more reliable than the erythrocyte sedimentation rate, and that the measurements of

viscosity appeared to be a promising method of investigation in rheumatic diseases.

Woodmansey and Wilson (7) have reported similar findings. While Race (8) believes that in the more severe clinical conditions the plasma viscosity test is superior to the erythrocyte sedimentation rate, he considers that the erythrocyte sedimentation rate may be the more sensitive in detecting the earliest changes in rheumatic disease.

The statistics of the present paper are based on the results obtained up to the end of February 1948, at Sunderland, and to the end of December 1947, at the Crossley Sanatorium (Table I).

So far as we know, the subject of plasma viscosity has not yet been discussed in the American literature: our results are, therefore, presented in order that readers in the United States can form an opinion on what we consider to be the most useful of the physical, non-specific laboratory estimations.

METHODS AND MATERIALS

I. Methods

The type of viscometer that we have used throughout our tests is accurate to about one part in 500, and requires only about 0.7 ml. plasma for each test. This is the Whittington viscometer, of which a full specification has already been published (4), together with the details of calibration and operation. Like the original large-capacity Ostwald type of instrument (of which it is a modification) this viscometer requires a waterbath and a stop-watch as additional apparatus.

As anticoagulants, our experience with sodium citrate solution, potassium oxalate, mixed oxalates and heparin leads us to believe that the present choice lies between the 3.8% aqueous solution of sodium citrate (1 volume solution: 4 volumes blood) and the Heller and Paul mixture (9) of potassium and ammonium oxalates as used for the Wintrobe erythrocyte sedimentation rate.

The range of normality with 3.8% sodium citrate solution is both absolutely and relatively narrower than with oxalate; thus the differentiation between normal and pathological is easier with the citrate technique. It has the disadvantage that the proportion of citrate solution to plasma depends on the volume of erythrocytes present since a given volume of whole blood is added to a fixed

TABLE I
Number of plasma viscosity estimations

Laboratory	(Anticoagulant) 3.8% Sodium Citrate Solution				(Anticoagulant) Heller & Paul's Oxalate Mixture				Total
	Normal	Pulmonary tuberculosis	Other	Total	Normal	Rheumatic disease	Other	Total	
Crossley Sanatorium, Frodsham	14	499	—	513	—	—	—	—	513
Royal Asylum, Montrose	19*	134*	268*	421*	9*	—	255*	264*	685*
Royal Infirmary, Sunderland	4†	23	37†	64†	47†	750	169†	966†	1,030†
Total	37	656	305	998*†	56	750	424	1,230*†	2,228*†

* 264 tests were performed using both Sodium Citrate and Mixed Oxalates.

† 27 tests were performed using both Sodium Citrate and Mixed Oxalates.

volume of citrate solution. It is therefore necessary to make an accurate hematocrit estimation followed by an empirical correction to the observed plasma viscosity, so as to allow for the proportion of citrate solution diluting the plasma in a given case.

The oxalate results are satisfactory for most clinical purposes. As the volume of blood to be added to the prepared bottle is not critical, it is certainly the method of choice where the collection of blood is undertaken in imperfect conditions, such as in a busy out-patient department. Also, it is convenient to estimate the plasma viscosity on a portion of a sample of blood removed for other hematological investigations.

When examining such colloidal solutions as plasma, serum, etc., the "viscosity" value depends on the dimensions of the particular individual viscometer. The differences between two viscometers with the same plasma may be considerable. It is essential therefore to standardize the viscometers to a common standard. The methods of standardization have been discussed elsewhere (4, 10).

Quarter-ounce (7 ml.) capped bottles are prepared for 5 ml. of blood by evaporating in an incubator 0.2 ml. of an aqueous solution containing 2% potassium oxalate and 3% ammonium oxalate. Five ml. (± 0.5 ml.) of venous blood, drawn with minimal stasis, is added. The contents are mixed by gentle rocking to avoid the formation of froth. In the laboratory the blood is centrifuged to separate the plasma. One ml. of the plasma is pipetted into a small evaporating dish, the viscometer is filled, and the viscosity is estimated as detailed previously (4). While it is preferable to complete the estimation as soon as possible, serial testing has shown that delay of as much

as five days in some instances has not altered the viscosity results.

When sodium citrate is used as the anticoagulant, 2 ml. of a 3.8% aqueous solution is pipetted into a 10-ml. volumetric flask or a centrifuge tube graduated at 10 ml. Venous blood is added exactly to the mark and gently mixed. A sample is removed for hematocrit estimation. The remainder is treated as for the oxalate blood above. An empirical correction has to be applied to this viscosity to make allowance for the variation in the plasma-citrate mixture due to anemia. A nomogram for this correction has been published (4).

On the basis of the complicated inter-relationship of the citrate plasma viscosity to the maximum citrate erythrocyte sedimentation rate the viscosity range has been divided into 11 zones. These zones are of special interest with reference to pleural effusions. The limits of the zones are in Table II.

II. Patient Material

(a) *Tuberculous patients*: Clinical grouping of the patients is based on a recent Ministry of Health circular (11) which can be briefly summarized as follows:

A-category: patients in whom tubercle bacilli have never been discovered.

B-category: patients in whom tubercle bacilli have been demonstrated at some time.

Each category is subdivided as follows:

Group 1. Slight or no constitutional disturbance: physical signs and radiological findings of very limited extent: no complications (tuberculous or other) of prognostic gravity.

TABLE II
Division of citrate plasma viscosity range into zones

Zone Number	1	2	3	4	5	6	7	8	9	10	11
Range	1.400 1.515	1.515 1.558	1.558 1.585	1.585 1.671	1.671 1.698	1.698 1.762	1.762 1.832	1.832 1.865	1.865 1.953	1.953 2.038	2.038 and over

TABLE III
The plasma viscosity in rheumatic conditions

Condition	Number of tests	Plasma viscosity		Average packed cell volume	Average Wintrobe E.S.R.	Average total plasma protein
		Usual range	Average of series			
Normal	56 (43)*	1.700-1.950 (1.700-1.950)	1.835 (1.853)	per cent 44.6 (42.9)	mm./hr. — —	g. per cent 7.12 (7.15)
Rheumatic fever						
Acute	16	2.250-2.900	2.449	36.1	45.3	7.80
Chronic	11	2.025-2.250	2.091	40.1	21.0	7.85
Rheumatoid arthritis†						
Residual deformity only	23 (8)	1.740-1.990 (1.740-1.990)	1.909 (1.909)	41.9 (41.8)	15.0 (19.8)	7.24 (7.40)
Chronic	387 (154)	1.950-2.300 (1.950-2.300)	2.119 (2.121)	42.2 (42.6)	24.7 (25.2)	7.77 (7.30)
Subacute	79 (22)	2.300-2.550 (2.300-2.550)	2.506 (2.513)	41.1 (40.8)	34.6 (36.1)	8.56 (8.50)
Acute	29 (16)	2.500-3.600 (2.500-3.400)	3.002 (2.897)	39.9 (38.8)	35.9 (38.8)	9.50 (9.54)
Infective polyarthritis (atrophic)‡						
Chronic	58	1.850-2.300	2.067	43.6	20.7	7.64
Subacute	8	2.450-2.700	2.550	38.4	30.4	8.44
Chronic hypertrophic osteo-arthritis§	27 (17)	1.875-2.075 (1.875-2.075)	1.929 (1.921)	43.9 (44.1)	13.4 (8.5)	7.38 (7.31)
Non-articular rheumatism	16 (10)	1.725-1.950 (1.725-1.950)	1.888 (1.896)	45.3 (46.3)	7.8 (6.7)	7.23 (7.34)
Still's disease	7	2.050-2.850	2.352	34.2	45.0	8.01
Gout	11	1.800-2.300	2.051	44.0	23.8	7.65
Atrophic spondylitis	14	1.750-2.300	1.983	44.1	13.0	7.40

* The figures within the parentheses are the statistics for the first 320 tests in Rheumatic Diseases, as previously published (Cowan and Harkness [6]).

† Rheumatoid arthritis—synonym, Atrophic polyarthritis.

‡ Infective polyarthritis (atrophic)—cases of atrophic polyarthritis where there is a definite focus of infection, e.g. in a nasal sinus, which is related to the arthritic condition.

§ Chronic osteo-arthritis—synonym, Hypertrophic, degenerative osteo-arthritis.

|| Non-articular rheumatism—synonym, Fibrositis, Bursitis, Panniculitis, Periarthritis, Myalgia, etc.

Group 3. Profound systemic disturbance or constitutional deterioration, and marked impairment of function, either local or general. All cases with grave tuberculous or non-tuberculous complications are placed in this group.

Group 2. All cases which cannot be placed in Group 1 or Group 3.

The Ministry of Health grouping is made on admission and it is not established practice to revise downward any such grouping in the case of a recovering patient. However, an A-category patient would be transferred to the B-category if tubercle bacilli were demonstrated at any time during the course of treatment.

(b) *Rheumatic patients*: These patients were classified as in Table III under "condition."

RESULTS

I. In Tuberculosis

Figure 1 shows the distribution of average viscosities in 330 tests on 289 individuals (including 37 controls). During the period since our last publication of results for pulmonary tuberculosis (4) we have performed 275 viscosity tests on tuberculous patients.¹

When interpreting Figure 1, it must be borne in mind that the viscosity values are only averages. If, for example, a sufficient number of B.2 sana-

¹ All results in this part are for citrate plasma.

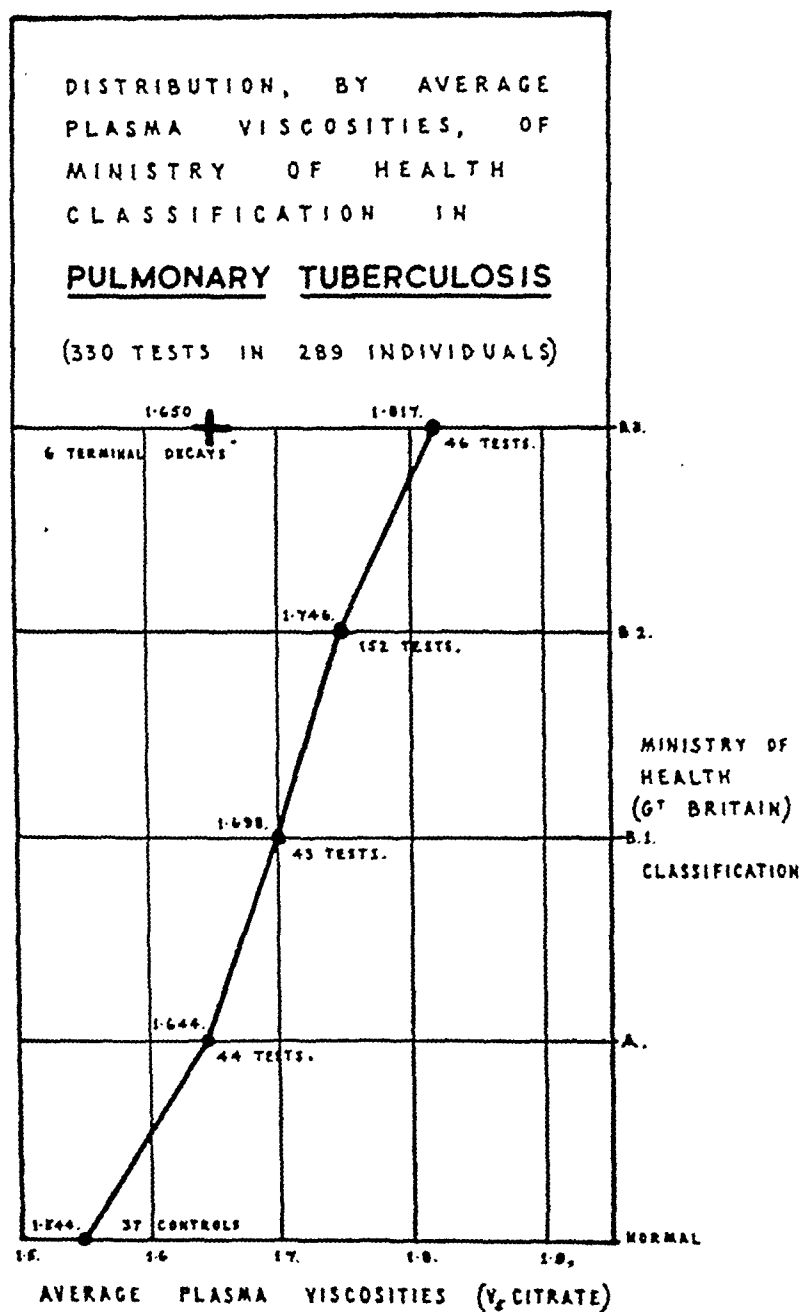


FIG. 1

torium cases be tested, the average of their viscosities will probably be found to be about 1.746. Nevertheless, the graph shows how, on the whole, the viscosity increases with the spread of disease, since the average viscosities rise fairly steadily from the normals to the most advanced (B.3) category.

The graph does not show, of course, how the viscosities of recovering patients ultimately return

to the normal range, but we now have sufficient experience to be able to state definitely that this is so in the patients discharged without physical signs.

Where the disease ends fatally, we have observed a terminal phase in which the final advances of the disease are accompanied by a fall in viscosity towards the normal range. This phenomenon has been termed "terminal decay."

In Figure 2 we show the distribution according to the citrate plasma viscosity zones of those cases with definite clinical and radiological evidence of the production of clear pleural fluid at the time when the blood was removed for the test. Forty-two tests on 32 patients are given: 22 (52%) are

in viscometric zone 7 (1.762 to 1.832), and 32 (76%) are in zones 6 to 8. These results suggest that zones 6 to 8 form a region in which it is highly probable that exudative cases will lie.

It has been our experience that if a pleural effusion be aspirated while the viscosity is in zone 7,

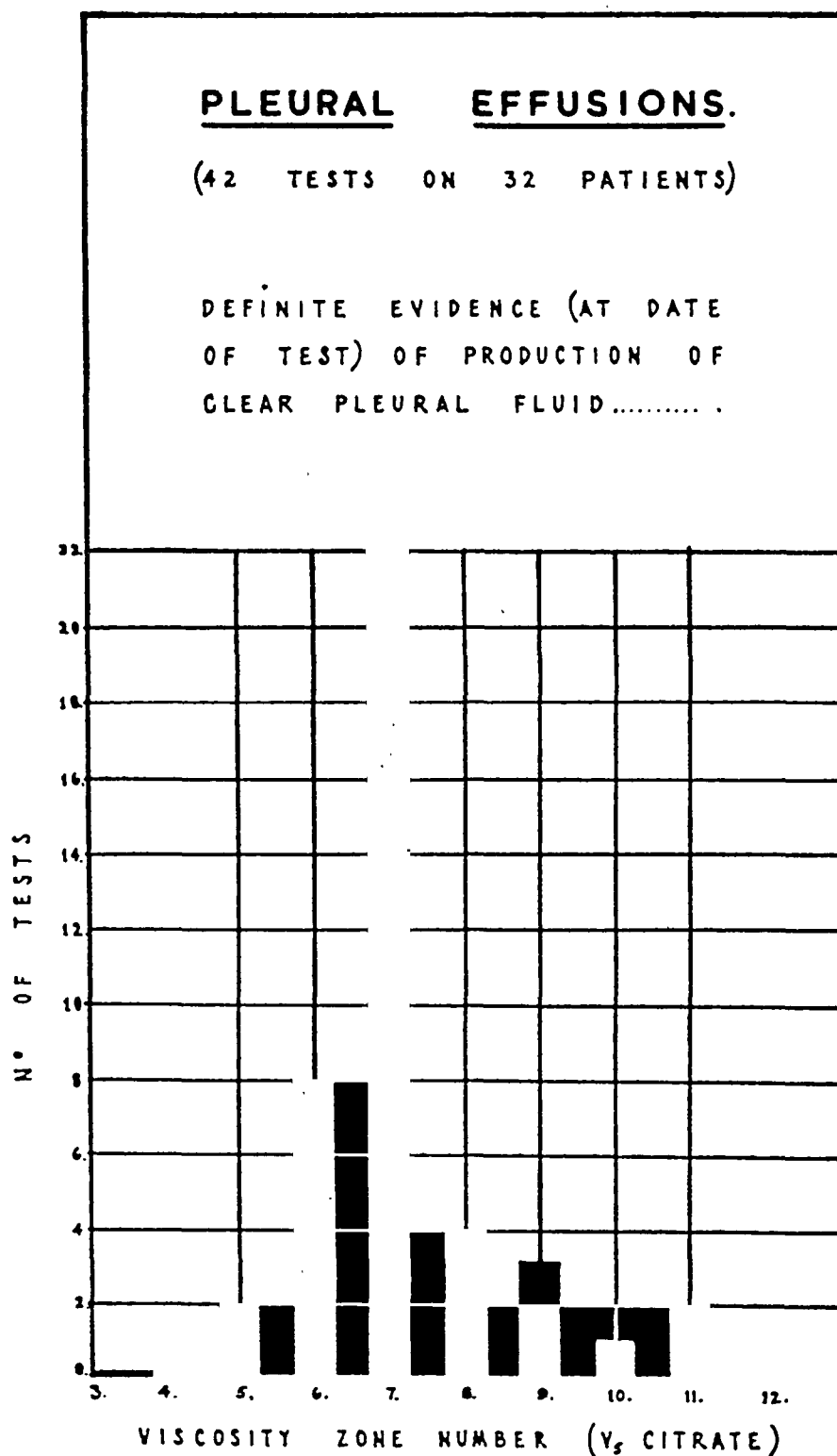
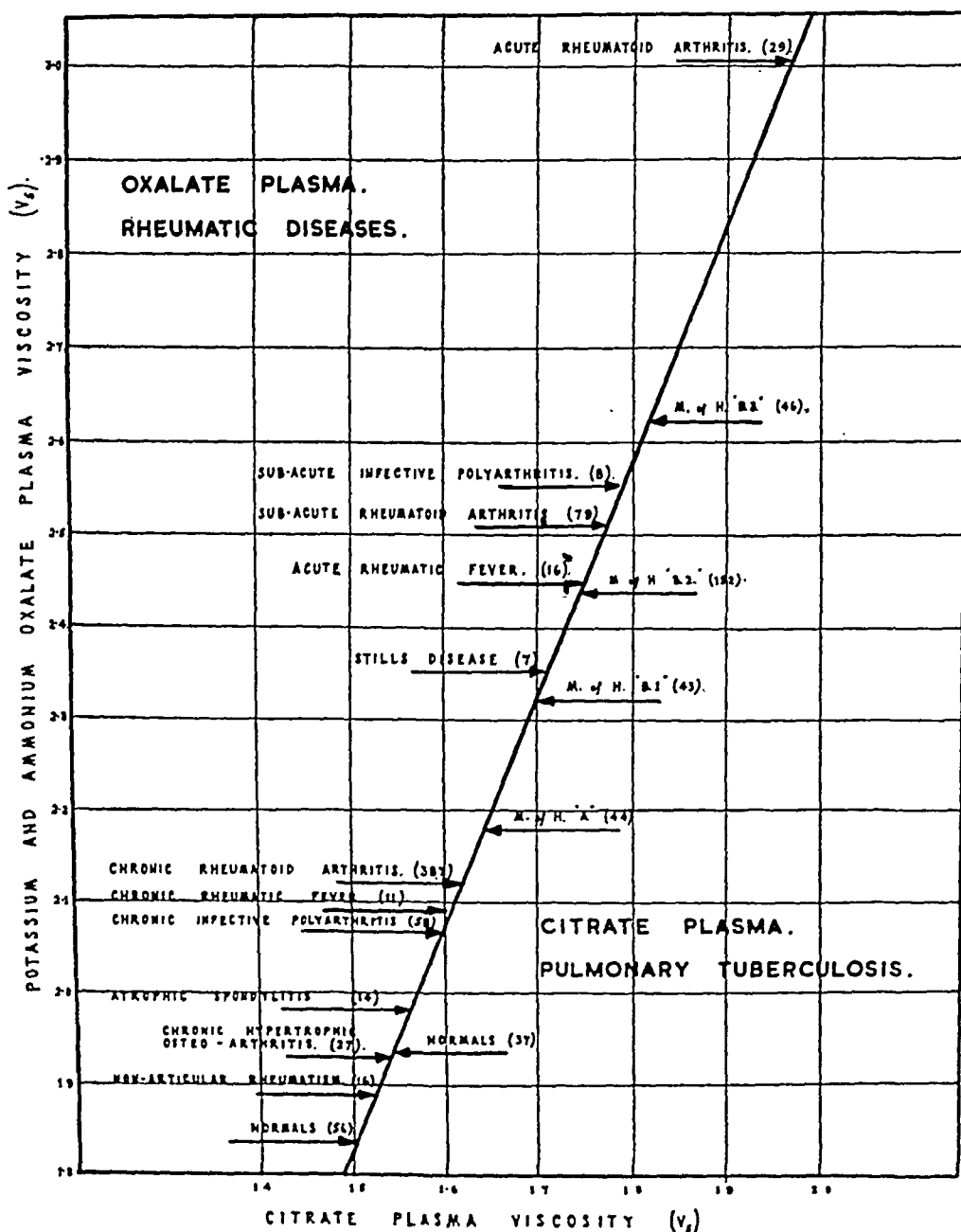


FIG. 2

the fluid reforms at once. In other cases whose viscosities are either above or below zones 6 to 8, fluid does not reform after aspiration, although if, later, the viscosity should return to the critical region, the fluid may reform.

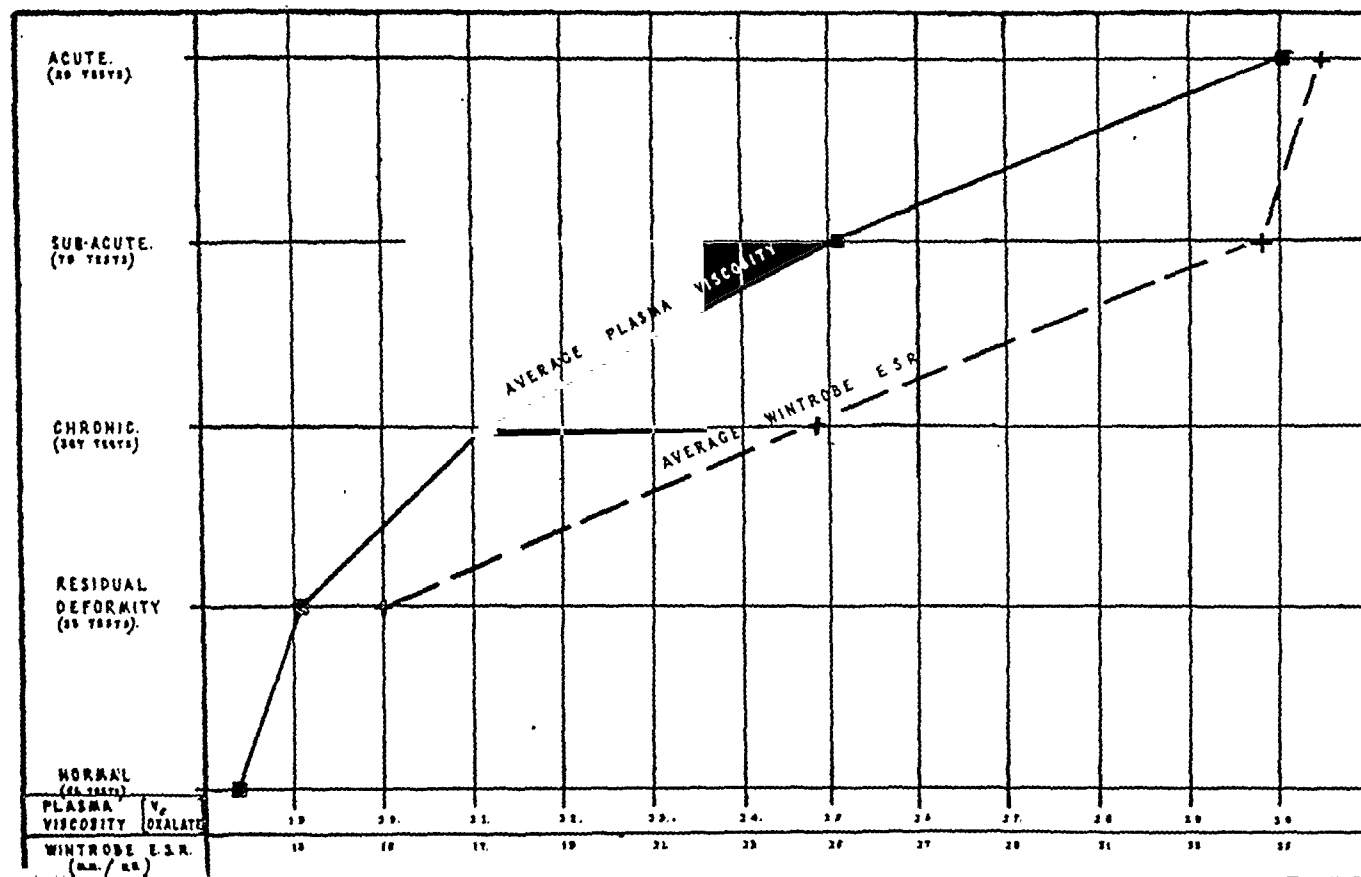
No physical explanation of this phenomenon can yet be attempted; but it appears to be of potential importance in the management of pleural effusions, especially as a complication of artificial pneumothorax.



DISTRIBUTION OF AVERAGE VISCOSITIES

DIFFERENT ANTICOAGULANTS IN THE TWO DISEASE GROUPS
NUMBER IN BRACKETS INDICATES NUMBER OF TESTS IN EACH GROUP

FIG. 3



AVERAGE PLASMA VISCOSITIES AND SEDIMENTATION RATES
(SHOWN AGAINST CLINICAL SEVERITIES) IN RHEUMATOID ARTHRITIS.

FIG. 4

It is of interest to report that in our only case of tuberculous pericarditis the fluid was formed when the plasma viscosity was in the critical zone 6 to 8 range.

II. In Rheumatic Disease ²

From our experience with more than 750 tests in rheumatic disease, the results can be summarized as follows:

(a) In every case in which the plasma viscosity was raised to an abnormal level (above 1.950) an underlying organic cause was ultimately found.

(b) In rheumatic fever, the plasma viscosity increased as the disease progressed and decreased during recovery. If, during recovery, the nature of the disease process changed from an acute to a chronic type, the change was shown by the cessation of fall or even by a rise in the plasma viscosity; the viscosity was a more sensitive index of this change than the temperature, pulse or erythrocyte sedimentation rate.

(c) In rheumatoid arthritis the increase in the plasma viscosity was roughly in proportion to the severity of the pathological changes in the locomotor system and to the systemic reaction.

The viscosity values changed with variation in the clinical condition. It required but slight changes clinically to produce significant alterations in the viscosity. With clinical improvement the viscosity values approached those found in normal persons.

In many instances where the plasma viscosity was raised, the erythrocyte sedimentation rate was within normal limits, yet clinically the disease process was active. Conversely, in many cases where residual deformity remained and there was no other indication of systemic upset, the erythrocyte sedimentation rate remained elevated while the viscosity had returned to the normal range.

Figures 3 and 4 present graphically the results given in Table III, indicating the increase of the plasma viscosity with increasing severity of the disease. Figure 4 emphasizes the inability of the

² All results in this part are for oxalate plasma.

erythrocyte sedimentation rate to differentiate between subacute and acute conditions.

(d) In cases of non-articular rheumatism where there appears to be no systemic reaction, the plasma viscosity values remain within normal limits.

(e) With chronic hypertrophic osteo-arthritis, there were indications of little or no systemic reaction and this was reflected in the viscosity results.

(f) The findings of the other groups of the rheumatic diseases are given in Table III and Figure 3.

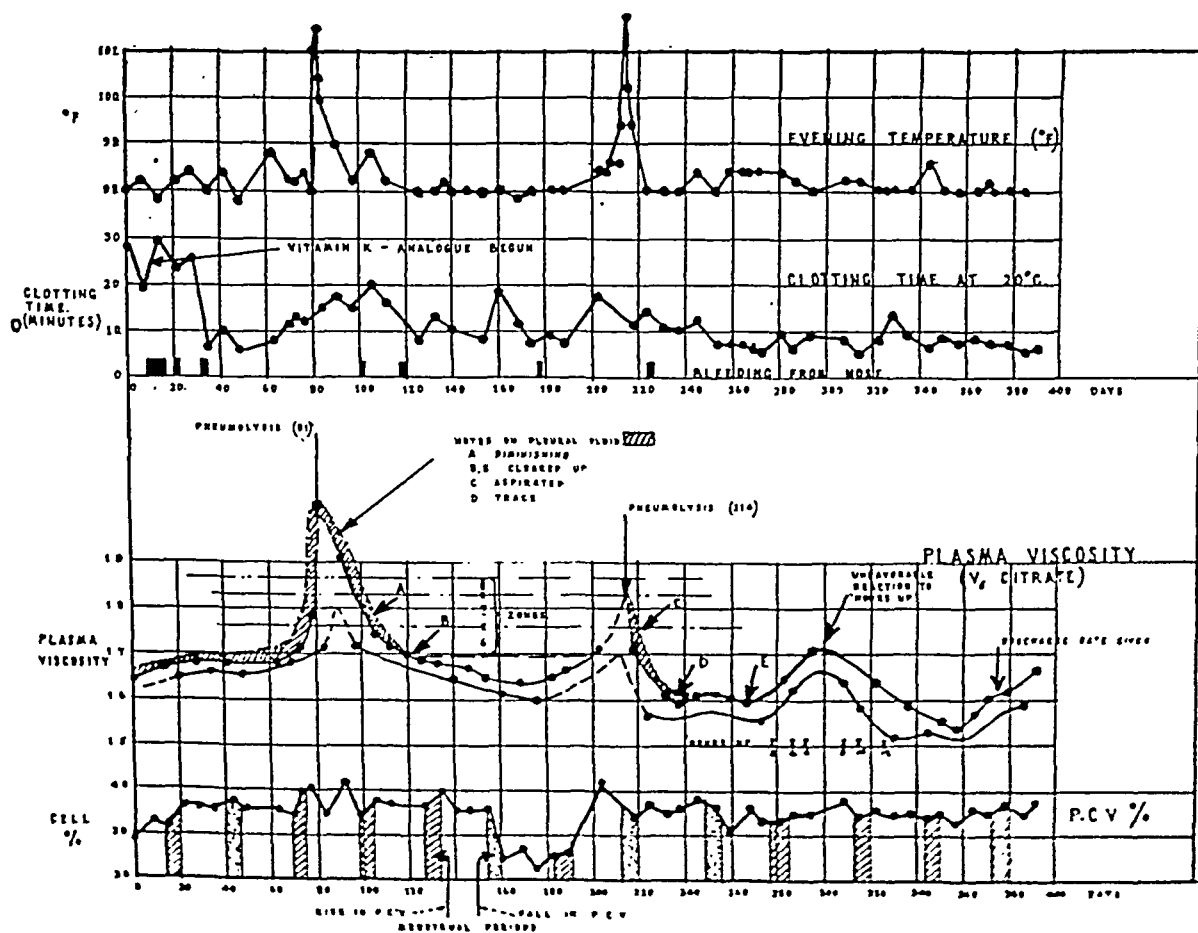
CASE HISTORIES

I. Tuberculosis

Case C. 280, a female, aged 30, was admitted to the Crossley Sanatorium on July 23, 1946, with extensive tuberculous infiltration of the upper third of left lung, with cavitation, and slight infiltration of right outer infraclavicular region.

This patient received routine sanatorium treatment for two months, resulting in improved general condition. Left artificial pneumothorax was induced (Day "minus 38"—chart reference). Following persistent nose-bleeding, the blood-clotting time was estimated and found to be considerably above normal. She was given 60 mgs. water-soluble Vitamin K analogue daily (Day "11"). At routine screening of the chest a minimal effusion in the left costo-phrenic angle was noted (Day "minus 4").

The various laboratory tests carried out, beginning at Day 0, are plotted in Figure 5. Day 81, left internal pneumolysis: three band-adhesions found, two divided and one to the third rib partially divided. On Day 122, the effusion had spontaneously cleared, the cavity remained patent. On Day 214, second internal pneumolysis: as much as possible of the third adhesion was divided. Left effusion developed at once after operation; and 60 ml. of clear fluid were aspirated on Day 221. This left a very small effusion in the costo-phrenic angle, which had cleared spontaneously by Day 269. The cavity was closed by Day 293 and thereafter clinical progress was uneventful.



SERIAL TESTS ON A TUBERCULOUS PATIENT

FIG. 5

This case illustrates several points which we feel are worth discussing. A glance at the chart shows three obvious peaks. All three peaks have definite clinical significance. Peaks 1 and 2 are related to internal pneumolysis, while the third peak is probably a reaction to increasing hours of exercise.

A most interesting feature of peak 1 is that the rise in viscosity was first noticed on Day 73, eight days before the operation, and this rise continued steeply on Day 77, as shown. The patient was informed some time between Days 69 and 73 that adhesion-section would be performed on Day 81; and fear of the proposed operation is suggested as the cause of the pre-operational rise. A similar explanation is offered for the pre-operational rise at peak 2.

In support of these observations, we would refer to the findings of Macfarlane and Biggs (12) who have investigated the occurrence of fibrinolysis associated with trauma. They found that in some cases "fear of impending operation" appeared to cause positive results. They also drew attention to parallel observations made by Latner (13) who recorded fibrinolysis in normal subjects during air-raids.

We feel that the rise shown by the last few readings may also have a psychological explanation, since the patient was notified on Day 374, after many months of sanatorium sojourn, of her actual "discharge date."

These phenomena appear to show that the plasma viscosity can be an extremely sensitive index of a patient's condition.

The occurrence of pleural effusion is shown hatched on the viscosity chart. The initial effusion was observed at Day "minus 4," so that we have no record of the viscosity reading at the time of production of fluid. The amount of fluid present as observed radiologically did not appear to increase after Day 0, despite the fact that the plasma viscosity passed through zones 6 to 8 prior to and following the pneumolysis. Later, however, as the viscosity fell further the effusion cleared spontaneously by Day 122. From this point until the second pneumolysis, the pleural cavity remained dry. Immediately after this operation when the viscosity was within zones 6 to 8, a second effusion occurred. This was aspirated on Day 221 when, according to the graph, the viscosity was in zone 5; and apart from the small residuum left at aspiration, no further accumulation of fluid took place. By Day 269, the pleural cavity was dry.

II. Rheumatic Diseases

1. Acute Rheumatic Fever. Three weeks before admission to the hospital, B. R., a male, aged 42 years, had a sore throat which was followed eight days later by elevated temperature, joint pains and other evidence of a classical attack of acute rheumatic fever. Salicylates were administered in doses sufficient to cause buzzing in the ears, with only partial response. The patient was admitted to the hospital on July 22, 1947.

Sodium salicylate internally and methyl salicylate locally to the painful joints were continued until August

13, 1947, when the sodium salicylate was discontinued (because of toxic symptoms) and replaced by calcium aspirin. The plasma viscosity and the erythrocyte sedimentation rate on August 12, 1947, showed a moderate reaction. The alteration in treatment was followed immediately by an acute recurrence of joint pains, elevation of temperature, sweating, etc., and this worsening was reflected in the increase of viscosity of August 18, 1947, while the erythrocyte sedimentation rate showed an appreciable reduction.

On August 17, 1947, the sodium and methyl salicylate were resumed. On August 23rd, while the patient was receiving large prophylactic doses of penicillin, some decayed teeth were removed. Thereafter improvement was continuous until his discharge on September 11, 1947 (to rest at home). Again the improvement was reflected in the plasma viscosity changes; the erythrocyte sedimentation rate rose inexplicably on August 25th, thereafter also mirroring the clinical improvement.

Date	P.C.V. (%)	Plasma viscosity	Plasma protein (g./100 ml.)	Maximum citrate E.S.R. (mm./hr.)
8-12-47	41.2	2.186	7.34	55.7
8-18-47	38.5	2.228	7.13	41.2
8-25-47	39.0	2.183	7.42	43.5
9- 4-47	41.4	2.107	7.24	35.1
9-11-47	41.3	1.997	6.91	28.8
1- 9-48	47.2	1.895	6.95	7.4

When the patient reported three months later to the out-patient department both tests had returned to normal values; there was no clinical indication of activity; there was no evidence of cardiac involvement by the disease.

The plasma viscosity here appears to be a useful index of the pathological processes occurring in the patient.

2. Chronic Rheumatoid Arthritis. O. G., a female, aged 25 years, gave birth to a normal healthy baby in July, 1946. Three months later she began to complain of pain and stiffness of the small joints of the fingers, and then of a tendency towards ulnar deviation of the fingers at the metacarpo-phalangeal joints. Gradually pain and swelling developed in both knees, and there was swelling of the right ankle joint.

There was fusiform swelling of the small joints of the fingers, ulnar deviation at the metacarpo-phalangeal joints, periarticular thickening of both wrists with limitation of dorsiflexion of the right wrist; both knees were hot and swollen, and there was limitation of flexion of the right knee by 20°; the right ankle was "puffy" and painful, although there was full range of movement. General medical examination revealed no focus of infection; urine showed no abnormality; skiagram of chest was negative. Skiagrams of the involved joints showed changes typical of rheumatoid (atrophic) arthritis. The picture was typical of a case of chronic rheumatoid arthritis of some months' duration.

She had been confined to bed at home for eight months prior to admission to hospital on July 2, 1947, when her blood test gave the following results:

Date	P.C.V. (%)	Plasma viscosity	Plasma protein (g./100 ml.)	Wintrobe E.S.R. (mm./1st hr.)
7-2-47	41.2	2.117	7.54	—

Treatment consisted of rest in bed, active physical therapy, procaine and lactic acid injections (Waugh [14]) to the affected joints, and a course of gold injections (myocrisin). She improved slowly, and by August 20, 1947, she was reported to be capable of standing alone and walking with assistance. The improvement was reflected in her next result:

9-9-47	39.6	2.038	7.45	—
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Treatment was continued. Ferrous sulphate tablets were given to combat the anaemia. Improvement was continuous; and she was discharged on September 30, 1947, walking unassisted.

9-30-47	40.4	1.974	7.16	—
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Three months later, at the beginning of December, she had an attack of influenza. This was followed by a flare-up of all the involved joints. The condition had deteriorated from one of temporary quiescence to one of marked activity, the change being shown also in the laboratory results.

12-22-47	43.4	2.326	8.46	28
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The patient rested in bed and a further course of physical therapy and myocrisin was given. At the end of the course the pain in the joints had diminished, and weight-bearing was again possible.

3-9-48	39.3	2.103	7.70	40
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The clinical impression at that time was that the disease condition was being controlled; the fall in the plasma viscosity supported this contention, in spite of the rise in the erythrocyte sedimentation rate. Out-patient treatment was continued. Unfortunately, on June 28th, she reported pain in the right hip which had not been affected previously; a skiagram of this joint showed changes typical of atrophic arthritis. The plasma viscosity reflected this increase in the activity of the disease, in keeping with the involvement of a new joint (a major one). On the other hand, the erythrocyte sedimentation rate decreased.

7-1-48	42.9	2.213	8.35	32
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3. *Chronic Hypertrophic Osteo-arthritis.* K. M., a female, aged 50 years, gave a history of pain and stiffness in both knees of six weeks' duration. She had had no other illnesses in her life and no history of injury was given.

There was marked periarticular thickening of both knees, and considerable quadriceps wasting. There was no increased local heat and no synovitis. Her fingers showed Heberden's nodes. Skiagrams of the knees showed the appearance typical of chronic hypertrophic osteo-arthritis. Her laboratory results were:

5-22-47	37.9	1.871	7.45	10
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Here, as is usually the case in chronic hypertrophic osteo-arthritis, the plasma viscosity was within normal limits.

4. *Non-Articular Rheumatism.* J. H., a male, aged 34 years, had chronic fibrositis of the upper back almost continuously following rheumatic fever at the age of 17 years. July 17, 1948, after three days in which an acute flare-up made it impossible for him to move his head or shoulders without acute pain, his laboratory findings were:

Date	P.C.V. (%)	Plasma viscosity	Plasma protein (g./100 ml.)	Wintrobe E.S.R. (mm./1st hr.)
7-17-48	45.2	1.849	7.31	—

DISCUSSION

The plasma viscosity test is non-specific. We feel, however, that it is valuable in following the progress of various rheumatic diseases as well as pulmonary tuberculosis. It depends almost entirely on changes in the plasma protein (especially the fibrinogen and globulin fractions), since ultrafiltrate experiments have shown that the viscosity of the ultrafiltrate fluid is practically constant.

If the liver is regarded as the organ which is chiefly responsible for the formation of these plasma proteins then the plasma viscosity is a measure of a change in a liver function, in response to a disease process in the body.

It is not surprising that in pulmonary tuberculosis the systemic reaction and hence the plasma viscosity should parallel the severity of the disease process. In rheumatoid arthritis also the plasma viscosity varies directly with changes in the joints and soft tissues. This emphasizes the general nature of rheumatoid arthritis and the fact that the changes in the joints are only one part of the whole disease process.

In striking contrast are the findings in hypertrophic osteo-arthritis—a localized condition with little systemic reaction—where marked changes can occur in the joints with very little alteration in the plasma viscosity. Similar conclusions can be made for the other rheumatic diseases. In non-articular rheumatism there is an absence of systemic reaction; and little change in the plasma viscosity. Increased viscosity does occur, however, in such systemic diseases as gout, Still's disease, infective polyarthritis² and atrophic spondylitis.

Since the plasma viscosity is affected only by the plasma proteins, the question arises as to whether it would not be more accurate and informative to

² See footnote, Table III.

measure the proteins themselves. Consideration of Table III and the individual case histories will show that the total plasma protein is not so sensitive or reliable an indication of the clinical changes as the plasma viscosity. The estimation of the plasma viscosity is technically much simpler and less open to error than the estimation of the protein fractions. Our experience with over 250 parallel estimations of the plasma viscosity and protein fractions has indicated clearly that the viscosity test is by far the more sensitive; the viscosity may have reached definitely abnormal levels when the fractions are still within the usual range of normal values. It is possible to find abnormal protein fractions associated with a normal viscosity, but many of these are cases of terminal decay, to which reference has already been made: in this instance, protein fractionation may be more informative than the viscosity test.

We have not been able to detect any uniform plasma protein pattern associated with plasma viscosities in zones 6 to 8, where pleural effusions tend to form. In addition to this zone there is another of great clinical significance which cannot be detected readily by protein fractionation. Into the range of 2.025 to 2.175 (oxalate) fall the majority of cases who are suffering from chronic conditions of all types—rheumatic fever, rheumatoid arthritis, infective polyarthritis, syphilis and pyogenic infections. If in the course of a disease two viscosity values, at a few weeks' interval, fall into this zone, it is highly probable that the disease is of a chronic nature; we have noted this happening on several occasions during recovery from acute rheumatic fever when the condition has taken on a chronic nature. The importance of the ability of the test to detect a change from an acute to a chronic process need not be stressed from the aspect of prognosis and treatment.

Although the plasma viscosity test and the erythrocyte sedimentation rate may both reflect changes in the plasma proteins, the results of the two tests may be dissimilar. Careful correlation of the results of these tests with the clinical condition has shown that the plasma viscosity is not only the more reliable but that the erythrocyte sedimentation rate can be definitely misleading. The superiority and the reliability of the plasma viscosity test become very obvious when individual patients have serial examinations during

the course of their illnesses. While it must be admitted that in some cases with clinically apparent early disease the erythrocyte sedimentation rate will begin to rise before the plasma viscosity starts to change, there have been instances in which an abnormally high erythrocyte sedimentation rate has been found in an apparently normal person and no cause for this increase has ever been found. In contrast is our experience with over 2500 plasma viscosity tests where in every case of a high viscosity value the cause of this increase was ultimately detected.

CONCLUSIONS

1. The plasma viscosity has been estimated in 656 tests on cases of pulmonary tuberculosis and in 750 tests on cases of rheumatic disease.
2. The results have been considered generally and some representative cases have been given in detail.
3. The plasma viscosity test is not specific. It depends on changes in the plasma proteins.
4. The plasma viscosity indicates systemic reaction to disease processes.
5. As an indication of the presence of and changes in the systemic reaction the test is sensitive and reliable.
6. The systemic reaction (and hence the plasma viscosity) mirrors the changes in the local lesions in pulmonary tuberculosis, rheumatic fever, and rheumatoid arthritis.
7. The formation of pleural effusions is usually associated with plasma viscosities within a narrow range of values.
8. Chronic disease processes are also associated with a narrow range of viscosity values.
9. The value of the plasma viscosity test to the clinicians treating pulmonary tuberculosis and rheumatic diseases is discussed.

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PROCEEDINGS OF THE FORTY-FIRST ANNUAL MEETING OF THE
AMERICAN SOCIETY FOR CLINICAL INVESTIGATION
HELD IN ATLANTIC CITY, N. J., MAY 2, 1949

READ BEFORE THE SCIENTIFIC SESSION

PRESIDENTIAL ADDRESS

CLINICAL RESEARCH AS A CAREER

By WESLEY W. SPINK

On May 10, 1909, Dr. S. J. Meltzer, the first President of this Society, addressed the members on the subject, "The Science of Clinical Medicine: What it Ought to be and the Men to Uphold it." In his introductory remarks he said, "The honor of the presidency of a society goes with the privilege of discussing some of the aims of that society." In discharging my responsibility as President, forty years after Dr. Meltzer, I am tempted to review the accomplishments of this Society, but such would be idle boasting. I would like to call your attention to the history of this Society by Dr. J. Harold Austin of Philadelphia which appears in the March, 1949 issue of the *Journal of Clinical Investigation*. It is well worth reading. Dr. Meltzer was deeply interested in the capable young individual who elected a career of clinical research. Since today, more than ever, young individuals are continuing to manifest an interest in clinical investigation, I would like to discuss with you some of the problems relating to their training.

The Clinical Investigator as a Clinician

Clinical investigation implies the critical study of human beings in sickness and in health. This suggests many ramifications of effort. While fruitful studies on human disease may be pursued within the confines of laboratory walls without the investigator ever coming in contact with patients, someone has had to define the problem or problems in disease that the patients present. It cannot be emphasized too strongly in this day of highly specialized techniques and laboratory research that successful clinical investigation is dependent upon a knowledge of disease in human beings. If one is setting out to resolve a problem in human disease, it is desirable to know as much as possible about the natural history of that disease. This information is not to be gleaned in an armchair, pouring over books and journals, but rather by the persistent observation of patients on the wards and in the dispensaries. The individual interested in a career of investigation should pursue further clinical training after completing his internship by selecting an appointment in which he will be given responsibility for the care of patients and in an institution where he will have the time and facilities to engage in research. Competent advisors should be readily available to aid him in his clinical activities and to lend direction to his investigations.

What attitude should the resident in clinical medicine interested in a career of research and academic medicine take toward certification by one of the specialty boards

of medicine? This is not the place to discuss the trend toward specialization in medicine as reflected by the specialty boards. But I see no valid reason why the resident should not fulfill the requirements of certification while he is obtaining his training. If he expects some day to have an academic appointment of any merit, in which he will be responsible for the clinical training of undergraduate and graduate medical students, he should at least possess that general knowledge of medicine which is necessary to pass the examinations of a specialty board. Furthermore, after he has had his formal residency training and has had an opportunity to engage in research activities, he may desire to go into the practice of medicine, or, much against his wishes, economic circumstances and obligations may make it mandatory that he enter into care of his own patients. Engaged in the private practice of medicine as a highly trained clinician, certification by a specialty board will be a decided advantage. However, it is difficult to lend a sympathetic ear to the individual whose primary interest in a residency is to fulfill the requirements of a specialty board and who applies for an appointment on a service devoted to teaching and clinical investigation with this purpose in mind.

The Clinical Investigator and the Basic Sciences

Clinical investigation today, contending as it does with the complexities of human disease and the assimilation of quantitative information, has narrowed the possibilities of carrying out fundamental research at the bedside of the patient. Highly technical knowledge is essential and the special tools of a basic discipline are frequently needed. The clinician may obtain help with his problem by turning to his colleagues in the basic sciences or to skilled laboratory technologists. Under these circumstances, he throws himself on the mercy of others for the accuracy of the acquired data and for their interpretation. It is difficult to evaluate laboratory results if one is unacquainted with the basic principles involved, and one cannot seek out the sources of error if he is ignorant of laboratory methods. Therefore, in the prosecution of clinical research, it is frequently necessary for the clinician to take time out and go into the laboratory. A year or two may be devoted to one of the basic sciences such as physiology, bacteriology, or pharmacology. Today it is not unusual for clinicians to concentrate on a review of the advances in the fields of chemistry or physics. A brilliant chapter in the history of clinical investigation includes modern cardiovascular surgery

achieved by the amalgamation of basic physiologic principles and improved surgical techniques worked out in the laboratory on lower animals. The surgical correction of coarctation of the aorta was not evolved by the method of trial and error on human subjects, but the techniques emanated from the laboratory after months of painstaking thought and effort.

The Clinical Investigator and the Research Team

One of the developments in recent years in clinical research has been the organization of personnel as a team in the prosecution of a problem. This tendency toward joint effort was accelerated during the last war. For many projects there are distinct advantages that accrue from pooling the abilities of several investigators. Among the disadvantages lurking in such an endeavor is the shackling of the independence of thought and initiative of young investigators. Any individual who wants to do original work in clinical research must be extremely discriminating in electing to participate in a large project lest his freedom of thought and action be smothered by the desires of his supervisors. This trend toward team research applies not only to the medical sciences but also to other fields.

The Clinical Investigator and the Preparation of Data for Publication

It is unfortunate that many investigators in the course of their training have not been given direction in communicating their results to others. While this applies also to the verbal presentation of data, I am particularly concerned with the preparation of manuscripts for publication. Such an endeavor often represents several years of work by the individual, and an institution may have expended thousands of dollars in giving that person an opportunity to carry out the research. And yet it is pathetic to see the results embodied in an inarticulate exposition. That young investigators are not being given guidance is manifested by the appeal made by the editors of the *Journal of Clinical Investigation* in the issue for September, 1948, when they cited the deficiencies of papers being submitted to them and asked that more diligence and care be given to the preparation of a manuscript. Some principal investigators are so engrossed with their own research and personal interests that they have neither the time nor the concern for aiding the young investigator in the assimilation of his data and preparing them for publication. The writing of a scientific paper is not easy, and there are no short cuts in preparing an accurate and readable report.

The Clinical Investigator and His Economic Status

Individuals who elect to spend three or more years as a resident in one of the clinical disciplines face the grim reality that the financial return at best is but sufficient to meet the basic needs of existence. It is to be regretted that this applies to those who are encouraged to extend this postgraduate period of training and continue on with clinical investigation. Considerable admiration

must be extended to the courage of the war veteran with a wife and children who has made the decision to stay with clinical research. Too often, able individuals find it utterly impossible to continue on with the stipends accorded them, and they drop out and go into more lucrative fields of medical endeavor, especially private practice. It has become quite obvious that medical schools, hospitals, and institutes of research cannot satisfactorily finance the medical program of graduate training in medicine. It is also apparent that private enterprise in general cannot continue to support medical research as in the past. Where, then, are the institutions charged with the responsibility of training clinical investigators to turn for financial aid? Major support today is coming from the Federal Government. The taxpayer is footing large expenditures of funds for medical education and research. Because of this postwar development, I would like to discuss briefly Federal aid that is coming from three sources. First, a most remarkable union has been made between medical education and the Veterans Administration. This joint enterprise has resulted in an excellent training program for residents in the various specialties, and it has encouraged sound clinical investigation. The end result has not only been of considerable aid to the medical schools in discharging their obligations for postgraduate medical training to the veterans, but the patients have benefited from the skilled medical care. If a large segment of clinical material is to be hospitalized under the Veterans Administration in the future as at the present time, it is absolutely essential that medical schools continue with this cooperative effort. But the union will only continue successfully if the same high standards set up by the Deans' Committees and the Veterans Administration continue in force. A second type of Federal aid to medical education and research is that manifested by the United States Public Health Service. These funds have been administered wisely and have supported many excellent research projects. In addition, the Public Health Service has fostered a modest but sound program of education and research for promising young individuals. A third type of Federal aid to education and research is that distributed by the military branches. It seems almost incredible that in a free society desperately seeking ways to achieve world peace, tremendous sums of money should be allocated to military agencies for applied and fundamental research, particularly when much of the research has no direct bearing on military activities. While freedom of thought and action in research has accompanied the Army and Navy grants for medical investigations, it should never be forgotten that anything earmarked military implies security and that, under these circumstances, freedom may be stifled overnight in the interests of "national security."

Some young investigators are sorely grieved, and perhaps rightly so, at the scanty financial returns afforded them during their formative years. If these individuals anticipate later on in life an income commensurate with their labor and contributions to medical research, they will be sadly disappointed. As the young investigator matures, and after he has established his ability as a

clinician and as a scientist, he is often faced with the serious decision as to whether he can continue in the field of medical research on a full-time basis. Too many times, economic circumstances demand that he compromise and attempt the blending of the private practice of medicine with clinical research. It is not my purpose to belabor the question of private practice versus the full-time clinical investigator, but this problem confronts most men who are attempting to develop and direct medical research. As he concluded his address to the first meeting of this Society, Dr. Meltzer stated, "Teaching medicine and furthering its science is a very serious business which ought to be carried on by men who are ready to devote all or most of their time to it—but let me tell you: beware of practice. It is a bewitching graveyard in which many a brain has been buried alive with no other compensation than a gilded tombstone." Twenty years later, another great figure in clinical research, Sir Thomas Lewis, wrote, "No investigator can be successful who allows, or is forced by circumstances to allow, solicitude for his patients to preoccupy his mind." While it would be highly desirable today for competent young men to continue on a full-time basis, we cannot escape the bald fact that hospitals and medical schools are able to support in this manner but a very limited number of these individuals. I am of the opinion that a man can carry on a restricted private practice and engage in clinical research. Under these conditions, a clinician is often a better teacher and, if clinical investigation means the study of human disease, patients coming to him for advice may be of considerable aid in the resolving of a clinical problem. But unfortunately, the well-trained clinician possessed of desirable personal characteristics is often sought after by other physicians and patients to the detriment of his research. A soaring income accompanied by an elevated social position frequently has more enchantment than the self-sacrifice and many frustrations that go with clinical research.

The Clinical Investigator and His Obligation to Society

The era in which we are living has been called the Scientific Age, and now we are told about the coming Atomic Age. Society with its "cold wars" and anxious tensions expects and needs leadership from scientists. While the attention of the public is centered chiefly on

those working in the fields of nuclear physics and electronics, the medical scientist has also grown in stature. Is the medical man prepared to assume his responsibility of leadership? One of the undesirable aspects of medical education is the emphasis placed upon the natural sciences and the scant attention given to the study of history, political economy, and the social sciences. When the student of medicine in this country finishes his undergraduate and postgraduate education, he has received the finest technologic training available in the world today. But he has not been educated for a position of leadership in society at large. He does not know, nor often does he appear to care, about the social, political, and economic changes disturbing the equilibrium of society. In his zeal to unravel a mystery of pathologic physiology at the bedside or in the laboratory, he has no time for the problems of the world with its clashing ideologies, opposing cultures, social hatreds, and nationalistic pride masking economic greed. The medical school curriculum could stand a little more emphasis upon social pathology, even at the expense of morbid anatomy. The young clinical investigator is the leader of medical education tomorrow. In concluding my remarks this morning, I would like to plead that the young medical scientists do not hide within their ivory towers of learning, oblivious of the community and its problems. As the investigator pursues his training, let him give time and thought to the immediate issues facing mankind. As he attempts to mold and influence the thinking and attitudes of medical students under him, let him remind his charges that the possessor of a medical degree is a humanitarian rather than merely a doctor dispensing his technologic skill to the highest bidder. Let him instill within his students the desire to acquire a social conscience. Let the young instructor inculcate upon his classes the fact that environmental factors are often more important in the genesis of human illness than organic changes. Finally, he knows and his students know that the distribution of medical care will undergo some changes. Let him beseech his students as they attempt to resolve this problem in their own minds that they utilize the experimental method in which conclusions are reached only after accurate data have been assimilated rather than on the basis of ignorance and selfishness.

Value of Blood and Urine Lactate Response to Physical Effort as an Index of Physical Fitness. DAVID I. ABRAMSON, and (by invitation) ROBERT W. KEETON, WARREN H. COLE, H. H. MITCHELL, J. M. DYNIEWICZ, M. C. GEPHARDT, S. J. PRESLEY, and W. R. KEARNEY, Chicago, Ill.

The effects of various work loads on the lactic acid in the blood and urine were studied in a group of patients in widely different states of physical fitness as judged by clinical standards. When all were subjected to the same small amount of work on a treadmill, the individuals in good physical condition showed much less of an increase in the blood lactate level, with the peak of the curve appearing sooner, than was observed in those in poor shape. The patients falling in categories between the two extremes demonstrated changes which could be correlated with their relative states of physical fitness. From the alterations noted in urine lactate content, the patients could only grossly be divided into good or poor performers.

When the individuals walked on the treadmill almost to the point of exhaustion, the variations in blood lactate response were such that further subdivisions with regard to gradations in physical fitness were made possible. In the patient in excellent shape, the blood lactate curve showed a peak, occurring at a relatively low point, early in the exercise and then a fall during the remainder of the walk; this continued into the post-exercise period. The individual in the next lower category did not demonstrate the fall during the latter part of exercise but a plateau instead. The patient in poor physical shape showed an ascending slope during exercise and a peak, occurring at a much higher level, late in the post-exercise period.

Following a major surgical procedure and its associated period of physical inactivity, many of the patients demonstrated alterations in the blood lactate curve which were interpreted as a decrement in the efficiency of their reaction to the work load.

It is concluded that the blood lactate response to exercise is a sensitive index of physical fitness.

Genetic Studies of 201 Persons with Hereditary Hypercholesterolemia (Thirty-five Families and Twenty-nine Individuals). DAVID ADLERSBERG and ALBERT D. PARETS (Introduced by Ernest P. Boas), New York, N. Y.

Continuing previous studies, 35 families (172 members) and 29 individual patients with hereditary hypercholesterolemia (Familial Xanthomatosis), totalling 201 persons, were investigated. Forty-nine patients (24 per cent) exhibited the full syndrome of hypercholesterolemia, coronary artery disease and one or more of the following: xanthoma tuberosum or tendinosum, xanthelasma and juvenile or senile corneal arcus; 122 had hypercholesterolemia (300 mg. per cent or more); 61 exhibited xanthelasma; 25 ex-

hibited xanthoma and 35, corneal arcus. Coronary artery disease was present in 80 patients (40 per cent). Among these, serum cholesterol was determined in 66 and was found elevated above 300 mg. per cent in 57. Thus, the two most frequent abnormalities encountered in these xanthoma families were hypercholesterolemia and coronary atherosclerosis. Xanthelasma and arcus were next in frequency, while xanthoma was found least often. Genetic analysis revealed that the inborn error of cholesterol metabolism was inherited as an incomplete dominant trait. The significance of these findings in the etiology of coronary artery disease is discussed.

The Role of Phospholipids in High-Lipid Sera in Cirrhosis and Nephrosis. EDWARD H. AHRENS, JR., and HENRY G. KUNKEL (Introduced by Sidney Rothbard), New York, N. Y.

In a large series of lipemic (milky) sera from 15 patients with nephrosis and non-lipemic high-lipid sera from 20 patients with biliary cirrhosis the ratio of phospholipids to total lipids (PL/TL) was found to be high in non-lipemic sera and low in lipemic sera. In fasting sera with total lipids ranging from 600 to 2,900 mg. per cent, PL/TL in non-lipemic sera was 0.29 or higher in 22 of 24 instances, whereas in lipemic sera PL/TL was 0.25 or lower in 15 of 19 instances. These data suggest that the clarity of high-lipid sera in biliary cirrhosis is explained by the characteristically large proportion of phospholipids, since these lipids are markedly hydrophilic by nature of their water-soluble side-chains. By this concept lipemia is due to a low proportion of hydrophilic lipids rather than, as commonly postulated, to an absolute increase of neutral fat.

This hypothesis was tested by destroying lecithin and sphingomyelin of serum enzymatically, using *Cl. welchii* lecithinase which splits phosphorylcholine from lecithin and sphingomyelin, depriving these lipids of their hydrophilic side-chains and leaving water-soluble diglycerides. Exposure of normal and high-lipid sera to this enzyme produced a lipemia indistinguishable grossly and microscopically from that of nephrosis, and the degree of turbidity measured spectrophotometrically was linearly proportional to the total lipid content.

Further studies showed that under certain conditions measurable enzymatic hydrolysis of serum phospholipids occurred before turbidity appeared or increased. Thus, it was demonstrated in high-lipid sera of nephrosis as well as cirrhosis that phospholipids exert an appreciable solubilizing effect upon grossly elevated hydrophobic lipids.

These data have possible application in studies of fat transport and deposition of fat in tissues.

A New Theory of Blood Coagulation with Reference to the Role of Prothrombin

tors. BENJAMIN ALEXANDER and (by invitation), GRETA LANDWEHR and ROBERT GOLDSTEIN, Boston, Mass.

Serum contains a substance which, arising during coagulation, can accelerate the conversion of prothrombin in plasma to thrombin by thromboplastin plus calcium. Many of its properties have been delineated, and it has been separated in a fraction comprising 20 mgm. of protein from 100 cc. of serum. Evidence indicates that it is a new clotting factor, distinct from Ac-globulin of Ware and Seegers, Factors V and VI of Owren, or Labile Factor of Quick.

The amount of accelerator which evolves during coagulation is related to the amount of prothrombin consumed. In hypoprothrombinemia—congenital, dicumarol induced, or resulting from liver disease—abnormally small amounts of accelerator are elaborated. Furthermore, the interaction of purified prothrombin preparations with thromboplastin and calcium yields substantial amounts of accelerator in addition to thrombin. Accordingly, it is proposed that prothrombin (M.W. 140,000) may be split during its conversion to give thrombin (M.W. 70,000) and the accelerator. This concept is suggested also by remarkable similarity in some respects between prothrombin and the accelerator.

The agent cannot accelerate prothrombin conversion in the absence of a non-prothrombin factor present in fresh plasma. Also, a short interval (approximately 2 minutes) is required for these components to interact. The plasma moiety is labile and appears identical with Labile Factor, Factor V and Plasma Ac-globulin, all of which, it is generally agreed, are probably the same.

On the basis of the above a new theory of blood coagulation is proposed: in the presence of thromboplastin and ionized calcium the prothrombin and serum is slowly cleaved to yield thrombin and serum accelerator. Under the influence of the serum accelerator plasma Ac-globulin (Factor V, Labile Factor) is converted to serum Ac-globulin which then accelerates the conversion of additional prothrombin to thrombin and more serum accelerator.

The overall reaction thus proceeds with ever increasing velocity until the prothrombin is almost entirely consumed. Reduction in serum accelerator after dicumarol administration rather than the hypoprothrombinemia per se may explain the efficacy of this drug in the prevention and treatment of thromboembolic disease.

Renal Function in Multiple Myeloma. JOHN B. ARMSTRONG (Introduced by Jerome Harris), Durham, N. C.

Patients with multiple myeloma frequently die in uremia. Pathological examination has emphasized blockage of the renal tubules by protein casts, as the cause of the renal failure. If this were true, functional tests should show approximately equal decrease in glomerular and tubular function.

Eight patients with a clinical diagnosis of multiple myeloma, confirmed by x-ray, bone biopsy, and other exami-

nations, were selected who showed no evidence of other kidney disease. All patients had normal blood pressures and NPN concentrations. The glomerular filtration rate (C_{IN}), effective renal plasma flow (C_{PAH}), and tubular secretory maximum (T_{MPAH}) were determined, each value being an average of two or more urine collection periods.

In each instance, all three functions were depressed to a lesser or greater degree. In three patients the tubular secretory maximum was much better preserved than glomerular filtration. The severity of renal impairment did not seem to correlate with the hematologic or serum protein abnormality, nor with the amount or duration of Bence Jones proteinuria. The renal functional impairment resulting from hemoglobin- and myoglobin-uria, studied experimentally by Corcoran and Page, showed greatly depressed tubular maxima, compared to the other renal functions, whereas in these patients the converse was the rule.

Tubular obstruction by precipitated protein does not adequately explain the renal impairment frequently found in patients with multiple myeloma. The pathological findings often show relatively little blockage. In one patient who died suddenly without uremia or dehydration, few casts were seen. It seems likely that cast formation may be part of the terminal picture of this disease and not the fundamental basis of the renal failure.

A Comparative Study of the Effects of Tetraethylammonium Chloride and Veratrum Viride on Blood Pressure of Normal and Toxemic Pregnancies. N. S. Assali and Albert A. Brust (Introduced by Eugene B. Ferris), Cincinnati, Ohio.

Humoral maintenance of toxemic hypertension as contrasted with largely neurogenic blood pressure control at term of normal pregnancy has been suggested by observations after autonomic blockade with tetraethylammonium chloride (TEAC). Application of this concept has been further investigated in the present study in which veratrum viride, frequently used in the treatment of toxemia, has been subjected to physiologic evaluation. Ten normal term pregnancies and 12 toxemic pregnancies have been studied with blood pressure assay using intravenous TEAC and veratrum.

Negligible blood pressure response to veratrum occurred at term and postpartum of normal pregnancy, whereas TEAC invariably produced marked blood pressure fall prepartum and minimal responses after delivery.

In toxemia, veratrum lowered systolic and diastolic levels by 50 per cent of the total blood pressure. TEAC administration to the same patients reduced the blood pressure by only 10 per cent. With postpartum recovery from toxemia the marked veratrum response disappeared. As veratrum blood pressure floors rose to levels comparable to those observed postpartum of normal pregnancy, the TEAC floors descended to the same range.

Veratrum side effects (bradycardia, epigastric burning, nausea and vomiting) occurred regularly, but the magnitude of the depressor response was independent of these vagal stimulation components of the drug action. Administration of TEAC at the height of veratrum action in-

variably abolished the side effects, yet the depressor action persisted.

The data: (1) Demonstrate diametrically opposing blood pressure effects of TEAC and veratrum in both normal pregnancy and toxemia. (2) Eliminate vagal stimulation as the cause of the marked depressor responses to veratrum in toxemia. (3) Indicate that the blood pressure of the toxemic hypertensive is consistently sensitive to veratrum. (4) Offer clues for discussion of possible modes of mediation of veratrum depressor responses in toxemia.

The Problem of Infection of Man with the Virus of Newcastle Disease of Chickens. F. B. BANG and DAVID T. KARZON (Introduced by Joseph L. Lilienthal Jr.), Baltimore, Md.

From two of three cases of acute conjunctivitis occurring in laboratory personnel working with the virus of Newcastle disease, we have isolated this virus on two occasions each. These individuals did not develop a high titer of neutralizing substance in their fresh sera. In a survey of eight other persons exposed in the laboratory but who had not had conjunctivitis, one was found whose fresh serum neutralizes more than one million lethal infectious doses for the chick embryo. After heating to 56° C. for one-half hour, this serum loses a large part of its activity, neutralizing less than one thousand lethal doses. When the serum is diluted, there is a very rapid drop in its titer of neutralizing substance. This appears to stand in contrast to a slower fall in titer when the immune serum of chickens convalescent from experimental Newcastle disease is similarly diluted. The substance has been consistently present in fresh samples of serum taken from this person during the past year. No substance of comparable activity has been found in the other sera tested. The nature of the heat labile component is under study.

A Quantitative Analysis of the Effects of Adrenocorticotrophic Hormone (ACTH) on Potassium (K) and Phosphorus (P) Metabolism. FREDERIC C. BARTTER, PAUL FOURMAN, ANNE P. FORBES, WILLIAM M. JEFFRIES and ELEANOR DEMPSEY (Introduced by Fuller Albright), Boston, Mass.

In man, ACTH affects K and P metabolism each by three established mechanisms. For K they are: (1) the loss of protoplasm, (2) the retention of extracellular fluid (e.c.f.), and (3) the specific effect of adrenal "salt" hormone in causing K loss. For P they are: (1) the loss of protoplasm, (2) the retention of e.c.f., and (3) the loss of bone (osteoporosis).

Balance studies with ACTH are reported. "Corrections" are applied to the K and P excretions for protoplasm, e.c.f., and bone changes. A fourth effect of ACTH on K and P is thereby unmasked, namely, K and P retention "on" ACTH, and K and P loss "off" ACTH. The deviations of K and P so revealed are highly correlated. This fourth mechanism affects K in the opposite direction to that of the "salt" hormone, and is not elicited by desoxycorticosterone alone.

We infer that this fourth effect represents passage of K and P into and out of cells, possibly associated with glycogenesis and glycogenolysis, respectively. We suggest that the K and P liberated when protoplasm is broken down are retained with glycogen. The net result is a transformation of protein energy to the more readily available glycogen energy.

Apparent Role of Shock in Production of Renal Damage Accompanying Hepatic Injury. JAMES H. BAXTER (Introduced by Benjamin M. Baker, Jr.), Baltimore, Md.

Rats fed diets containing pyridine regularly developed acute hepatic necrosis; the animals which survived long enough developed cirrhosis. Before death, the animals usually appeared pale and were cold to the touch. The livers of animals autopsied in the terminal stages of necrosis were engorged with dark blood. There was marked obstruction to perfusion of fluid through the livers. Bleeding from the rectum sometimes occurred. Disturbances of hepatorenal vasotropic factors (Shorr), in addition to obstruction and trapping of blood in the liver, may have contributed to the production of shock.

Many of the rats fed pyridine also developed renal injury. Degenerative changes and sometimes necrosis occurred in the convoluted tubules. Regenerative changes followed, with the formation of cellular buds or masses in the region of the straight segments. Generalized or localized dilatation of tubules with flattening of epithelium was regularly observed, and areas of cortical scarring and atrophy resulted when length of survival permitted.

Liver injury frequently occurred in animals without kidney injury. However, kidney injury was seldom observed prior to the occurrence of extensive liver injury, even though some animals remained on the diets for weeks without developing liver injury. Furthermore, the most extensive renal injury occurred in animals which remained in shock for considerable periods or which survived recurrent episodes of shock. It appeared likely that the renal injury was at least in part due to shock, resulting in impairment of cortical circulation with anoxia of tubular cells, oliguria, and increased concentrations of protein in the urine. Similar mechanisms may participate in the production of renal injury under a wide variety of circumstances.

Preliminary Studies on an Indirect Method for Determining Portal Pressures. WILLIAM B. BEAN and (by invitation) WILLIAM D. PAUL and MURRAY FRANKLIN, Iowa City, Iowa.

There are circumstances in which it is desirable to know whether or not pressure in the portal vein is elevated. Previous observations on portal pressures have been confined to experimental animals except for a few direct measurements obtained during abdominal operations. We have approached the problem in normal persons and those with clinical signs of cirrhosis of the liver by observing through the proctoscope or sigmoidoscope changes in color of the mucosal surfaces with measured increments of intraluminal pressure.

The escape of air upwards in the colon is prevented by inserting an inflatable rubber bag attached to a tube. The sigmoidoscope is connected with a water manometer and by T-tube to a rubber bulb pump and small air reservoir so that air pressure within the rectum and sigmoid can be varied at will and measured readily. Subjects are prepared by cleansing enemas. The rubber bag is inserted, the proctoscope introduced and the bag is inflated. The glass window with adaptor for communicating with the manometer and pressure reservoir are adjusted. With the patient in the left lateral (Sim's) position, the anatomical center of the liver is approximately on the same level as the observed mucosa. Pressure must be increased slowly to reduce intestinal movements and cramps. At levels between 10 and 20 cm. of water, the pink color fades and many vessels are seen against the pale background. Between 40 and 60 cm. the caliber of the vessels narrows. With manual pressure over the liver, the level at which blanching occurs is elevated to 30 cm. or more. Similar levels have been noted in persons with portal hypertension and cirrhosis. The method may be useful in evaluating pain thresholds in the lower bowel and the acute effects of drugs.

Myofilaments and Myofibrils of Cardiac Muscle. H. STANLEY BENNETT, Seattle, Wash.

Electron micrographs have been obtained of frog cardiac muscle elements fixed in formaldehyde or trichloroacetic acid and fragmented with blender and homogenizer. The preparations have yielded pictures of myofibrils, which prove to be packed with parallel myofilaments similar to those found in striated skeletal myofibrils by Hall, Jakus, and Schmitt. The cardiac muscle myofilaments are slightly thicker than those of skeletal muscle, averaging 240-260 Å in diameter, and show an axial repeating period of about 400 Å when unstretched. As in skeletal muscle, the filaments appear to be indefinitely long, extending through many sarcomeres. Evidence of fine structure within the 400 Å period can be seen, but the material does not permit definitive analysis of such details. The filaments are capable of being stretched readily and fortuitously during fragmentation or on the supporting film, so that periodicities from 375 Å to about 750 Å may be measured, depending on the degree of stretching. Electron micrographs of cardiac muscle myofibrils show the various bands visible with the light microscope, but the bands are less distinct than in skeletal muscle. The Z-band is about 0.15 μ wide in cardiac muscle.

The Effect of a Cation Exchange Resin on Electrolyte Balance and Its Use in Edematous States. EUGENE Y. BERGER, LESLIE IRWIN, BENJAMIN ROSENBERG and ROSLYN JACKENTHAL (Introduced by Alfred E. Cohn), New York, N. Y.

An exchange resin, Liquonex CRW, which removes cations from solution in exchange for hydrogen was fed to a subject on a constant diet. An electrolyte balance study was conducted for three weeks before, five weeks during, and three weeks following the administration of the resin. Approximately 80 per cent of dietary sodium and 70 per

cent of dietary potassium appeared in the stool. There was no evidence of a calcium deficiency. A compensated acidosis was induced by the liberation of hydrogen ions in exchange for sodium ions. The excess chloride, phosphate, and sulphate anions were balanced in the kidneys by formation of ammonia.

Fixation of sodium in the gut is equivalent to restricting sodium in the diet; hydrogen ion release is the equivalent of giving ammonium chloride or an acid ash diet. Both of these procedures are commonly used to help prevent edema formation. Therefore, two patients suffering from heart failure and edema and one from cirrhosis of the liver and ascites were fed the resin. In the two cases of congestive heart failure on a diet containing 6 grams of salt daily, the use of resin replaced the need for mercurial diuretics and salt restriction. In the patient with cirrhosis of the liver on a similar diet, the ascitic fluid disappeared in 22 days without the use of mercurial diuretics. A well marked but still compensated acidosis was produced in the normal individual and in the patient suffering from cirrhosis of the liver. These observations indicate that a cation exchange resin can restrict the absorption of sodium from the intestine. Use of the resin may therefore become important whenever it is desirable to maintain a high protein low sodium diet.

Studies in the Renal Mechanisms for the Excretion of Potassium. ROBERT W. BERLINER, THOMAS J. KENNEDY, JR. and JAMES G. HILTON (Introduced by A. B. Gutman), New York, N. Y.

When large amounts of potassium salts are administered to normal dogs by constant intravenous infusion, the amount excreted in the urine may exceed that filtered by as much as 80 per cent. Exact reproduction of such experiments is mechanically difficult in humans, but potassium clearances greater than glomerular filtration rate have been observed in normal man, and in normal and hypertensive man on the rice diet.

The coexistence of a secretory mechanism for excretion of a substance ordinarily filtered and reabsorbed has not previously been observed. The paradoxical behavior of K⁺, capable of moving across the tubular epithelium in opposite directions, makes evaluation of the relative magnitude of the reabsorptive and secretory mechanisms difficult since only the resultant can be examined. Efforts to evaluate the roles of these two opposing mechanisms have been made chiefly in dogs.

Chronic administration of moderate doses of potassium by inducing tolerance, facilitates excretion of sudden large loads of this ion at more rapid rates and at lower plasma concentrations than in unprepared dogs.

Infusion of potassium with non-reabsorbable anions such as ferrocyanide or thiosulfate regularly results in ratios of excreted to filtered higher than with reabsorbable chloride. Moreover, in such experiments, the minimal secreted potassium is so much larger than the anions which could possibly have been secreted that cation exchange must have occurred. Hypothetical mechanisms, the overall result of which is ionic exchange, are discussed.

When infused sodium thiosulfate or ferrocyanide is progressively replaced by the corresponding potassium salt, change from net reabsorption to net secretion of potassium is first associated with increased excretion of chloride and bicarbonate, and, later, reduced excretion of sodium. This probably indicates that excretion of large amounts of potassium is initiated by depression of reabsorption and enhanced by cation exchange.

Studies in Hypothermia: Part I—Oxygen Consumption, Transport and Utilization in Dogs at Low Body Temperature. W. G. BIGELOW, W. K. LINDSAY, R. C. HARRISON, R. A. GORDON and W. F. GREENWOOD (Introduced by James A. Dauphinee), Toronto, Canada.

This is the report of the first of a series of studies dealing with the physiological effects produced in dogs by general hypothermia.

Twenty-seven mongrel dogs were cooled to body temperatures of 20° C. or lower, by being placed in a controlled temperature room at -10° C. Oxygen consumption was determined in the first experiments by analysis of expired air collected in a Douglas bag and in the later ones by the use of a modified Tissot spirometer. Arterial and venous oxygen saturations and carbon dioxide contents were measured at intervals. The dogs were revived by placing them in a water bath at 42° C.

One hundred and twenty valid determinations of oxygen consumption during the process of cooling and rewarming are reported. Early in the study it was found essential to eliminate all shivering and to maintain normal arterial oxygen consumption. Anesthesia was used to accomplish the former, artificial respiration the latter. Oxygen consumption was found to decrease steadily and in a relatively constant manner at the rate of 5 per cent per degree Centigrade of temperature lowering, and to increase at a similar rate during rewarming.

The determination of arterio-venous oxygen differences revealed that, at least down to 18° C. and in the presence of a satisfactory heart action, the circulation in these cooled dogs was adequate to transport oxygen to the tissues.

The finding that the increase of oxygen consumption on rewarming paralleled the decrease on cooling, together with the fact that the general deportment and response of the dogs that survived showed no evidence of tissue hypoxia, indicates that tissues in the cold state are capable of oxygen utilization.

Generalized hypothermia under the conditions of this study reduces tissue oxygen consumption without producing significant tissue hypoxia.

Coronary Blood Flow, Cardiac Oxygen Consumption and Efficiency in Man. R. J. BING, M. HAMMOND, J. HANDELSMAN, S. R. POWERS, F. SPENCER, J. ECKENHOFF, W. GOODALE, J. HAFKENSCHIEL and S. KETY (Introduced by Alfred Blalock), Baltimore, Md. and Philadelphia, Pa.

The coronary blood flow and oxygen consumption per 100 grams of left ventricular tissue have been studied in

30 individuals, by combining the nitrous oxide method with coronary sinus catheterization. Left ventricular efficiency was calculated for normal heart muscle. In hypertrophy, only low values for efficiency can be considered significant, since total left ventricular weight cannot be estimated. Tests were performed on normal individuals, and on patients with anemia, essential hypertension and coarctation. Four patients in failure were also studied.

In the normal, left ventricular flow averaged 65 cc./100 grams/minute, left ventricular oxygen extraction 12 vols. per cent and left ventricular oxygen consumption 7.8 cc./100 grams/minute. The normal left ventricular efficiency averaged 23 per cent. In anemia, the coronary flow was increased, left ventricular extraction and oxygen consumption/100 grams/minute were reduced. In essential hypertension coronary flow/100 grams/minute, left ventricular oxygen extraction and oxygen consumption/100 grams were normal. In coarctation coronary flow/100 grams/minute, AV difference and oxygen consumption/100 grams/minute were increased. In failure the coronary flow/100 grams/minute was reduced and the oxygen extraction slightly increased. The oxygen consumption/100 grams/minute was normal. These findings indicate that 1, chronic increase in load results in no change in oxygen consumption per unit weight but in a total increase in oxygen consumption due to hypertrophy. 2, The cardiac efficiency in failure is low.

Characteristics of Visceral Sensation in Man, as Observed in Normal Subjects and Patients with Unilateral Sympathectomy. J. R. BINGHAM (by invitation), F. J. INGELFINGER and (by invitation) R. H. SMITHWICK, Boston, Mass.

Deep somatic pain in man can be differentiated from abdominal visceral pain. In 5 subjects, deep somatic pain was produced by injecting the intraspinal ligaments and the rectus abdominis with hypertonic saline. Before, during, and after deep somatic pain so produced, a balloon was distended in various parts of the duodenum and jejunum. The two types of pain were perceived to be clearly different. These observations do not support the statement of Lewis that deep somatic and abdominal visceral pain are identical.

After unilateral sympathectomy, pain produced by distending the gut is referred to the unoperated side. This observation was unequivocal in the 10 patients studied. The degree of lateralization appeared to vary with the site of stimulation. Greatest lateralization occurred with duodenal or biliary tract pain, less with high jejunal, and least with low jejunal or high ileal pain. This observation may explain the contradictory findings of others.

After unilateral sympathectomy, the pain threshold is elevated. In 4 patients (2 subjected to intestinal, and 2 to biliary tract distention), almost double the amount of pain stimulus was required after, as was needed before sympathectomy. In 5 other patients, studied only post-operatively, the pain threshold was elevated as compared with the normal average. This observation may explain why unilateral sympathectomy sometimes relieves visceral pain arising from a bilaterally innervated organ.

The common duct is bilaterally innervated. Distention of the common duct produced pain in one patient with a right unilateral, and in one patient with a left unilateral sympathectomy. This observation, confirming Ray's, emphasizes that complete desensitization of the biliary tract cannot be achieved by right sympathectomy.

Metabolic Fate of Infused Erythrocytes in Man. F. ROSS BIRKILL, MARY A. MALONEY and STANLEY M. LEVENSON (Introduced by John B. Youmans), Chicago, Ill.

The metabolic fate of infused erythrocytes in adults made significantly polycythemic by transfusions has not been known. Only inadequate and incomplete information has been available on erythrocyte survival and bone marrow function in such individuals. Data on these points were obtained in two normal adult males on a constant diet receiving serologically indentifiable, fresh, separated erythrocytes. Observations were made on body weight, nitrogen, potassium, and phosphorus balances; circulating plasma protein and erythrocyte mass; erythrocyte survival; sternal bone marrow; reticulocyte counts; serum bilirubin concentration; urobilinogen excretion; and liver function.

Survival time of the infused erythrocytes was not shortened. The infused red cell mass decreased at a normal, expected rate of 0.8 per cent per day. Concomitantly, the mass of the recipient's own erythrocytes declined at a rate of 0.4 to 0.8 per cent per day in direct proportion to the amount of the infusions. This progressive fall in the subject's own erythrocyte mass was probably due to erythropoietic depression rather than to abnormally increased destruction. This is suggested by the normal survival of the infused erythrocytes and by urobilinogen excretion consistent with breakdown of the total red cell mass at a normal, not an increased, rate. There was a direct linear relationship between the extent of bone marrow depression and the degree of induced polycythemia.

A slow, steady excretion of 0.5 to 1.0 grams of nitrogen per day (above the control equilibrium value) began shortly after the infusions and continued for one month. The total extra nitrogen excreted was roughly equal to the total nitrogen content of the infused erythrocytes. However, this excess nitrogen derived only in small part from the infused red cells. The greater part could be accounted for almost completely by nitrogen diverted from normal erythrocyte synthesis as a result of marrow depression.

The Effect of Increased Intra-Abdominal Pressure Upon Renal Excretion of Water, Sodium and Potassium in Normal Human Subjects and in Patients with Diabetes Insipidus. S. E. BRADLEY, and (by invitation) G. H. MUDGE, W. D. BLAKE and P. ALPHONSE, New York, N. Y.

Elevation of intra-abdominal pressure in man by application of a pneumatic girdle under a pressure of 80 mm. Hg has been shown to depress glomerular filtration, renal blood flow, and maximal tubular glucose reabsorption and diodrast excretion. The output of urine is greatly reduced and the urinary concentrations of such substances

as mannitol and inulin increase. Further studies were undertaken to assess the role of the antidiuretic hormone of the pituitary in this phenomenon and to determine whether electrolyte retention also occurred.

Mannitol, p-aminohippurate (PAH), sodium, and potassium clearances were determined simultaneously in six normal human subjects and in five patients with uncontrolled diabetes insipidus before, during, and after abdominal compression. In all but in two normal subjects and one patient with diabetes insipidus, glomerular filtration (as mannitol clearance) and renal blood flow (PAH clearance) decreased significantly, and the excretion of sodium, potassium, and water was regularly reduced. These responses were identical in both groups of subjects. Sodium and water output usually fell more than filtration, whereas changes in potassium excretion appeared to parallel changes in filtration. In most instances, the urinary concentration of sodium decreased during compression. All values tended to return toward the control levels following release of pressure. Increased intra-abdominal pressure appears to induce a renal retention of water and electrolytes primarily as a result of a reduction in glomerular filtration, independently of the production of antidiuretic hormone. Augmented tubular re-absorption of water and sodium may account for disparities between changes in filtration and in sodium and water excretion observed in some subjects.

Tetralogy of Anomalous Pulmonary Drainage, Auricular Septal Defect, Right Ventricular Hypertrophy and Patent Ductus Arteriosus. ROBERT A. BRUCE, FRANK W. LOVEJOY, EARLE B. MAHONEY, GEORGE B. BROTHERS, PAUL N. G. YU and RAYMOND PEARSON (Introduced by Nolan L. Kaltreider), Rochester, N. Y.

Since patent ductus arteriosus is usually amenable to surgery, and ligation of an anomalous pulmonary vein has been recommended, the combination of both lesions together with auricular septal defect and right ventricular hypertrophy presents a complicated clinical problem. Two out of 12 possible patients reported in the literature lived for 19 and 27 years. We have studied 2 cyanotic young women, one of whom was proved to have this tetralogy at autopsy, and the other had similar findings by catheterization. The first had frequent major hemoptyses for which she was subjected to ligation of an anomalous right pulmonary vein demonstrated by catheterization. She died of pulmonary edema, immediately following ligation of the anomalous vein. The second had marked clubbing but no hemoptyses. The x-ray findings in both were a relatively small heart despite enlargement of the right auricle and prominence of the pulmonary conus and of vascular markings in the lungs. Each had right axis deviation electrocardiographically. Exercise studies showed transient paradoxical response in the respiratory efficiency attributed to the shunts. The right ventricular pressure considerably exceeded the systemic pressure in both patients, and in one catheterization of the left ventricle demonstrated that the duration of systole was less than that of either the right ventricle or systemic pulse. This was in accord with Brody's hypothesis that the right ventricle

could supply a major part of the systemic circulation through the ductus. Our experience shows that cyanosis contra-indicates ligation of an anomalous pulmonary vein whenever it is demonstrated to be draining into the right heart and that in this tetralogy patency of the ductus is essential. Slides of the laboratory data, anatomical findings in the first patient, and cinefluorograms (courtesy of Dr. Sibley Watson) of both are shown to demonstrate this particular tetralogy of congenital cyanotic heart disease.

The Lack of Correlation between Glomerular Filtration Rate, and Serum Electrolyte Concentration Changes, Urinary Electrolyte Excretion, or Edema Formation Following Sodium Loads in Subjects with Normal Kidneys, Glomerulonephritis, and the Nephrotic Syndrome. CHARLES H. BURNETT, (by invitation) BELTON A. BURNOWS and (by invitation) ROBERT R. COMMONS, Boston, Mass.

The effects of sodium loads administered intravenously to subjects with normal renal function, and with glomerulonephritis in different stages were studied. Normal responses to 200 to 400 milliequivalents of sodium (as 0.5 M sodium bicarbonate) included: serum concentrations, sodium increased, potassium slightly but definitely decreased; urinary excretion, sodium and potassium increased; percentage excretion of filtered ion (calculated from C_{15}), sodium slightly and potassium markedly increased; ratio of reabsorbate concentration to serum concentration (R/S), sodium remained close to unity, potassium fell (as much as 25 per cent). The direction of these changes was consistent despite individual variations in filtration rate and urine flow.

A different sequence resulted in the nephrotic syndrome, with normal or only moderately low glomerular filtration rates; sodium rose or was unchanged in the serum, urinary excretion failed to increase, percentage excretion of filtrate remained low, but R_{Na}/S_{Na} was unchanged. Further edema always resulted. With one exception, the effects on potassium metabolism were identical with, or more marked than, those occurring in normals.

In glomerulonephritis, without edema or heart failure, both the normal and nephrotic responses occurred. Ability to excrete the sodium load diminished as C_{15} fell; but excess sodium was better excreted than in nephrosis, and edema never resulted. The potassium responses were accentuated in that percentage excretion of filtrate often exceeded 100 (suggesting renal tubular secretion of this ion).

It is concluded, that since the rate of sodium excretion could not be correlated uniformly with either the glomerular filtration rate or tubular reabsorption of sodium, the mechanism of edema formation remains undisclosed in the diseases studied. It is suggested that the almost invariable change in potassium metabolism resulting from sodium administration may represent an important factor in the maintenance of salt and water equilibrium.

The Incidence of Liver Involvement in Chronic Amoebiasis. RICHARD B. CAPPS and (by invitation) ALFRED BENNETT, Chicago, Ill.

The incidence of liver involvement in chronic amoebiasis is of interest both because of its bearing on treatment and also because of its role in the production of symptoms. In order to provide both a sensitive and a specific method of establishing the presence of amoebic hepatitis, the response to emetine of a group of liver function tests was studied. Observations were made on a series of 15 consecutive cases of chronic amoebiasis. All cases had symptoms but in no instance was fever present. Liver function tests including the cephalin cholesterol flocculation, thymol turbidity, serum alkaline phosphatase, serum bilirubin, bromsulphalein, 24 hour urine urobilinogen and urine coproporphyrin were performed before and after a course of emetine. In 11 cases the initial tests indicated mild liver dysfunction. Following emetine the abnormal findings returned to normal promptly or dropped sharply in almost all instances indicating a specific therapeutic response. In 10 of these 11 cases liver tenderness and symptoms consistent with hepatitis were present initially and also responded to emetine.

We believe that these findings indicate that a specific hepatitis is present in a large percentage of cases of chronic amoebiasis and is responsible for many of the symptoms. Unless other agents can be shown to be effective emetine should probably be used routinely in all cases of amoebiasis. Further studies are being conducted.

The Golgi Apparatus in the Cells of Synovial Tissue and Related Structures. PEDRO M. CATOGGIO and L. RAYMOND MORRISON (Introduced by Charles L. Short), Boston, Mass.

A study was made of the relationship between the size, configuration and localization of the Golgi element and the histological findings in tissue from joints, bursae and ganglia removed at operation from seventy-five patients. In some instances the relationship to the joint fluid was also studied.

The material was prepared according to the Da Fano technique. Tissue from some of the cases was also studied by other methods.

The clinical diagnoses were: normal joints, acute traumatic arthritis, chronic traumatic arthritis and degenerative joint disease, rheumatoid arthritis, miscellaneous joint disease, bursitis, ganglion.

Examination of the sections showed a well-developed Golgi element in the synovial cells. In some cases it was small, granular or simple and delicately looped and was often indifferently located within the cytoplasm of small cells; presumably in the "resting state." This appearance was usually associated with apparently normal synovial tissue. In other instances the Golgi apparatus was large, complex and lay in relation to the free border of the cell and was presumably "active." These specimens showed various synovial tissue alterations: increased number of synovial cells, larger cells, villi and often perivascular round-cell infiltration.

If increase in size and complexity of the Golgi apparatus is evidence of increased activity of the cells, the above findings suggest that the synovial cells in rheumatoid arthritis are often more active than in normal

joints. In degenerative joint disease the activity of these cells appears to be between that in rheumatoid arthritis and that in normal joints.

In the small number of joint fluids available, comparison of the fluid characteristics with the appearance of the Golgi element in the tissue cells does not provide adequate evidence to aid in determining whether or not synovial cells produce mucin.

Simultaneous Determination of Cardiac Output by the Acetylene and Direct Fick (Right Heart Catheterization) Procedures. CARLETON B. CHAPMAN, HENRY L. TAYLOR, CRAIG BORDEN, AUSTIN HENSCHER, and ANCEL KEYS (by invitation) and RICHARD V. EBERT, Minneapolis, Minn.

Nineteen simultaneous measurements of the cardiac output by the acetylene and direct Fick (right heart catheterization) techniques were carried out on 7 normal subjects. The Grollman formula was used for acetylene determinations. The mean cardiac indexes were 2.47 ± 0.56 (acetylene), and 3.27 ± 0.78 (direct Fick) liters per square meter per minute. When plotted against each other, the results obtained by the 2 methods showed a definitely linear relationship ($r = 0.87$). Statistical analysis showed that the small deviation from a perfectly linear relationship was due to individual method errors. If the acetylene output (x) is known, the direct Fick output (y) can be calculated from the formula $y = 1.1682x + 0.7420$, the standard error of the estimate being ± 0.41 . The Grollman formula, therefore, can be made to yield results which closely approximate those obtained by the direct Fick procedure simply by altering the numerical constant.

In order to determine how much, if any, of the predictable difference between results of the 2 methods is due to recirculation of acetylene, 30 samples of pulmonary arterial blood were taken at from 9 to 55 seconds after acetylene entered the pulmonary alveoli. When the partial pressure of acetylene in the blood was plotted against time, recirculation began between 10 and 20 seconds after beginning of rebreathing and progressed in a linear fashion. For the interval between the first and second gas samples (9 to 19 seconds), the calculated mean partial pressure of acetylene in pulmonary arterial blood was 3.5 mm. Hg or about 5 per cent of the mean partial pressure of acetylene in the alveoli (61.5 mm. Hg). This confirms the view that such recirculation as occurs during the first 20 seconds after acetylene enters the alveoli is not enough seriously to affect the validity of the method.

Some Cardiac and Extracardiac Effects of Digitalis Glycosides. DON W. CHAPMAN and CARROLL A. HANDLEY (Introduced by James A. Greene), Houston, Texas.

Studies were undertaken to ascertain what relationship, if any, existed between the intravenous administration of digitalis glycosides and the serum concentrations of potassium and sodium and their effect on the electrocardiographic tracings; hematocrite; the plasma volume; and the extracellular fluid.

Digitoxin and digoxin were employed in concentrations from 0.1 to 0.2 mg./kg. body weight in twenty-seven dogs. Serial electrocardiograms were taken from lead 2 and blood samples were drawn at hourly intervals for the estimation of serum potassium and sodium. Sodium thiocyanate and T1824 were employed to ascertain changes in extracellular fluid and plasma volume at four to ten-hour intervals. Hematocrite determination was by the Wintrobe method.

When digitalis was administered in small doses so that relatively minor or no electrocardiographic effects were obtained, in general, there was but little change in the other studies. In larger doses an approximate qualitative relationship between the increasing height of the serum potassium and the electrocardiographic alterations, as seen in potassium intoxication, were noted. The initial changes observed were an increase in the amplitude of the T waves, followed by a decreased size of the R waves, and an increased amplitude of the S wave. In some, there was a disappearance of R waves with a depressed RST segment or a widening of the QRS complex. In others a partial or complete auricular arrest may have preceded the development of intraventricular block. Ectopic rhythms such as single or multiple premature ventricular contractions may be seen. However, in some instances, no direct correlation was noted with the height of the serum potassium. Marked increases in the hematocrite, a lowering of the plasma volume, and an increase in the extracellular fluid was observed when there were marked changes in the electrocardiographic tracings.

Multiple-Balloon-Kymographic Recording of Variations in Motility of the Upper Small Intestinal Tract in Man, Emphasizing the Need of Long Control Observations for the Adequate Evaluation of Drug Action. WILLIAM P. CHAPMAN, (by invitation) EIRWYN N. ROWLANDS, and CHESTER M. JONES, Boston, Mass.

Knowledge of spontaneous variations in intestinal motility in healthy subjects observed over long periods was regarded as essential for the evaluation of drugs, particularly when given orally. Fourteen studies averaging four hours in duration were made in twelve subjects, all except one being healthy young adults. Motility was recorded from the duodenum and upper jejunum using a four-lumen tube with balloons spaced at four-inch intervals. Placebos without verbal suggestion were administered on an average of fifty-one minutes after the start of the recording. Relative changes in contractions and "tone" or sustained tension of the intestine were assessed by visual inspection of the records and by polar planimeter measurements of surface areas beneath the tracings.

With individual exceptions, the group showed during the second, third, and fourth hours of observation a decline in peristaltic contractions fluctuating from thirty to thirty-seven per cent. Sudden, striking decreases in activity lasting from five to twenty minutes followed the spontaneous occurrence of spasms. Segmental waves, though difficult to assess, seemed to follow the course of peristaltic waves. "Tone" varied with alterations in the types of contractions and tended to increase slightly

with the passage of time. Most individuals showed a specificity of pattern as regards frequency, amplitude and duration of contractions and "tone."

Without a full appreciation of the spontaneous decline in contractions during long observation periods, the tendency of contractions to be temporarily abolished or decreased following spasms and individual motility variations, it is impossible to evaluate adequately the pharmacologic effects of drugs on intestinal motility. This is particularly true of drugs administered orally. Multiple balloon recording permitted simultaneous assessment of adjacent intestinal segments. Changes in motility appeared not to be conditioned by the administration of placebo as employed in this study. Infrequency of pain prevented its correlation with motility patterns.

Effects of Salt and Protein Restriction on Blood Pressure and Renal Hemodynamics in Hypertensive Patients.

H. CHASIS and (by invitation) W. GOLDRING, E. BREED, A. BOLOMEY and H. W. SMITH, New York, N. Y.

Twelve patients with essential hypertension were selected at random. Observations were made on blood pressure, glomerular filtration rate, renal blood flow and maximal tubular excretory capacity. The patients were maintained on a salt and protein restricted diet identical in composition with the rice diet suggested by Kempner, for from 15 to 98 days. Renal function measurements were made in 10 of the patients; in 9 the rate of glomerular filtration was reduced, in 8 the renal blood flow was decreased, and in 6 the maximal tubular excretory capacity was reduced. In 10 of the 12 patients no lowering of blood pressure was effected by the restricted diet used in this study. In the remaining two patients decrease in blood pressure levels of 24 and 22 mm. Hg systolic and 14 and 14 mm. Hg diastolic occurred. However, in view of the spontaneous variability of blood pressure levels, crudity of the sphygmomanometric method and difficulty in selection of a datum of reference during the control period, we are unwilling to accept as significant such relatively slight and transient reductions as indication of effectiveness of the dietary regime employed in this study.

In view of the adverse effects observed on renal hemodynamics and the transient and insignificant reduction of blood pressure we conclude that salt and protein restriction was ineffective therapy in the 12 hypertensive patients studied.

Studies on the Initiation of Coagulation by Surface Contact. C. LOCKARD CONLEY, ROBERT C. HARTMANN and JOHN S. LALLEY (Introduced by G. Canby Robinson), Baltimore, Md.

Using silicone-treated apparatus blood was obtained from normal human subjects without the use of anticoagulants. The formed elements were removed by high-speed centrifugation at low temperatures. Direct microscopic examination of the undiluted plasma revealed as a rule less than 10 platelets per cubic millimeter. In some instances no platelets were seen. Plasmas prepared in this manner regularly clotted within a few minutes at 37° C.

following the addition of ground glass. Platelet extracts prepared by grinding washed platelets in glass containers also brought about coagulation. However, the concentrated platelet extracts did not cause coagulation to occur more rapidly than did ground glass when added to these essentially platelet-free plasmas. The rate of clot formation following the addition of ground glass was related to the amount of glass added and was independent of the number of platelets or quantity of platelet extract present. The amount of prothrombin converted during coagulation seemed directly related to the amount of platelet material available. When plasma was clotted by ground glass in the virtual absence of platelets, prothrombin consumption seemed negligible. Unlike normal plasma, hemophilic plasma deprived of its platelets failed to clot on contact with glass.

Normal platelet-deficient plasma could be kept in silicone-treated tubes at 37° C. for relatively long periods of time without spontaneous coagulation even though clotting occurred promptly following the addition of ground glass. Although fresh normal plasma invariably clotted on contact with glass, portions of this plasma stored for 24 hours at 2° C. in some instances failed to clot on the addition of ground glass.

These observations suggest that the initiation of coagulation by surface contact may be brought about by alterations produced in the plasma itself and not necessarily by platelet changes.

Effects of Intravenous Reduced Glutathione (GSH) Upon Glycosuria and Hyperglycemia Induced in Man by Adrenocorticotrophic Hormone (ACTH). JEROME W. CONN and (by invitation) LAWRENCE H. LOUIS and MARGARET W. JOHNSTON, Ann Arbor, Mich.

We have reported that sustained glycosuria and hyperglycemia can be produced in man by administration of ACTH and that these manifestations of a developing diabetic state are accompanied by a decreasing concentration of blood glutathione. We have hypothesized that a purine metabolite having an alloxan-like effect is responsible for reducing the intracellular availability of free sulfhydryl groups (necessary for normal function of many enzyme systems), causing functional abnormalities within the islet-beta-cells as well as blocking normal glucose utilization peripherally. In addition to hyperglycemia, ACTH decreases renal tubular reabsorption of glucose, presumably as the result of inhibition of an enzyme within the tubular epithelium.

A thirty day metabolic balance study was made to determine whether or not administration of large amounts of GSH would reverse (during continued ACTH administration) an already established hyperglycemia and glycosuria produced in a normal man. GSH in large amounts was given on the fourth day of a six day ACTH period. Renal glycosuria occurred on the first day of ACTH. Hyperglycemia began on the second day and continued with increasing intensity throughout the entire ACTH period except during the interval when GSH was administered. This reversal was dramatic but transitory, lasting for one to two hours after each injection of GSH.

During these periods the urine was free of sugar. Most striking were (1) sharp elevation of the renal threshold and (2) a fall of the blood sugar level.

Not all of the metabolic effects of ACTH were reversed by GSH. This means that corticoid production was not blocked but that improved performance occurred in those systems requiring free S-H groups for their normal function. Among other reversed effects noted following GSH was a most profound change in the number and character and the peripheral blood cells.

All of these sharp reversals produced by GSH during ACTH administration strengthen our hypothesis and imply important hormonal influences upon enzyme activity.

Observations on the Hemolytic Mechanism in Paroxysmal Nocturnal Hemoglobinuria. WILLIAM B. CROSBY (Introduced by William Dameshek), Boston, Mass.

The red cells in paroxysmal nocturnal hemoglobinuria (PNH) are easily hemolyzed by the action of a heat-labile substance present in normal human plasma. This substance is similar in many respects to complement. Like complement, its hemolytic activity is blocked by heparin, although the amount of heparin needed for this inhibition is somewhat less than that needed to inhibit complement. Titers of 1 : 4,000 will almost completely block the hemolytic system of PNH *in vitro*.

Protamine, a potent heparin antagonist, prevents this heparin inhibition. Yet alone in the hemolytic system, protamine acts like heparin and inhibits hemolysis.

Titration with protamine or heparin in the PNH hemolytic system yields in each case, a sigmoid curve which breaks upward at about 1 : 3,000 and levels off at about 1 : 8,000. When cell suspensions containing the two antagonists in equal concentration are mixed, protamine seems much the stronger. Four parts of heparin cause no inhibition of hemolysis in the presence of one part of protamine.

A patient with PNH was given intravenously fifty mg. of heparin. This at once reduced the ability of his plasma to hemolyze his cells when incubated *in vitro*. During the ensuing six hours the hemolytic potency of his plasma gradually recovered. To a second patient, 80 mg. of heparin were given at the time of a transfusion in the hope of preventing a transfusion reaction to which she was prone. But a violent hemolytic reaction followed. In both of these patients the hemolytic mechanism was blocked *in vitro* by heparin.

Protamine and heparin are antagonistic by virtue of powerful and opposed electrostatic charges. Each alone is capable of inhibiting hemolysis in the hemolytic system in PNH. When both are present together in the hemolytic system they inhibit one another, and hemolysis occurs. Their effect is probably due to inhibition of a complement-like substance present in normal human plasma.

Primary Aseptic Meningitis: A Clinical Syndrome of Diverse Etiology. EDWARD C. CURNEN and (by invitation) JOSEPH L. MELNICK, and ERNEST W. SHAW, New Haven, Conn.

During the summer of 1948 a number of children and young adults were admitted to hospitals in Connecticut and Rhode Island with the clinical syndrome of primary aseptic meningitis. In most instances a diagnosis of poliomyelitis was made. These cases considerably exceeded in number those with paralytic poliomyelitis. The clinical and laboratory findings were studied in selected patients with representative non-paralytic illnesses.

Prominent initial symptoms were fever, headache, and vomiting. Nuchal rigidity and stiffness of the back were common but transitory complaints. The leukocyte counts approximated normal. The cerebrospinal fluid revealed a pleocytosis, usually less than 500 and predominantly lymphocytic; the protein content was normal or slightly elevated.

The course of the illness was brief and uncomplicated. All patients recovered completely. In patients with this syndrome, differentiation of non-paralytic poliomyelitis from mild non-paralytic illnesses due to other causes usually could not be made from clinical findings alone.

Attempts were made to recover the causative agent from representative patients with primary aseptic meningitis. Suspensions of stool from 2 of 14 patients tested induced poliomyelitis in monkeys. Similar suspensions injected into suckling mice resulted in the recovery of a filterable agent resembling that reported by Dalldorf and Sickles. Separate strains were obtained from 5 patients. Attempts to recover this agent from 9 other patients with aseptic meningitis (including the 2 whose stools induced poliomyelitis in monkeys), from 2 patients who developed paralytic poliomyelitis in 1948 in Connecticut, and from 20 patients with other illnesses were unsuccessful. Further efforts are in progress to characterize this agent, to correlate its occurrence with clinical and serological findings, and to determine whether infection by it represents a distinct disease entity.

Physiologic, Metabolic, and Electrolytic Balance Studies of Hypertensive Patients While on the Rice Diet. H. CURRENS, E. A. S. REID, E. A. MACLACHLAN and M. L. TERRY (by invitation) and A. M. BUTLER and P. D. WHITE, Boston, Mass.

In an effort to determine the *modus operandi* of the rice diet two men (one had previously undergone sympathectomy) were studied before and after instituting dietary therapy. The following pertinent observations were made.

The blood pressure fell in one patient after two weeks (174/126 to 160/111) and the other after three months (166/112 to 141/87) on the diet. The addition of nine grams of salt per day to the latter resulted in a rise (164/103).

A negative balance was observed for sodium, chloride, calcium, phosphorus, and magnesium on the diet. One patient had a positive potassium balance of about 20 milliequivalents per day. The serum concentration of these electrolytes remained essentially the same. The negative nitrogen balance was approximately five grams per day during the first week on the diet decreasing to one gram

after three months. The serum albumin fell significantly after three months.

An abrupt fall of the basal metabolic rate of about 12 points was observed after beginning the diet. No significant weight loss was noted. The insensible weight loss per day remained the same.

The plasma volume as measured by the Evans blue dye before and after the institution of the diet was found to remain constant.

Renal clearance tests revealed a subnormal glomerular filtration rate during the control period which decreased after starting the diet. No change was noted in the effective renal plasma flow. The tubular excretory mass was observed to decrease on the diet.

The change in blood pressure observed was not associated with a significant change in plasma volume or serum electrolytes. The addition of sodium chloride resulted in a rise in blood pressure. A persistent negative nitrogen balance was noted.

Metabolic Studies in Infants During Recovering from Vomiting. T. S. DANOWSKI and (by invitation) L. GREENMAN, J. H. PETERS, R. GOW and F. MATEER, Pittsburgh, Pa.

Six infants with pyloric stenosis, together with 1 other with vomiting and diarrhea of undiagnosed origin, have been studied during convalescence by the balance method. The majority had characteristic hypochloremia and alkalosis. Azotemia was present in one. At the start of the individual studies, but following therapy with solutions of saline and glucose, 4 had abnormally low concentrations of serum potassium. The initial serum sodium ranged between 123 and 152 milliequivalents per liter.

Oral intake was then completely withheld, without further vomiting, until the obstruction had been relieved. During this time the patients received parenteral glucose solutions, small amounts of saline, and, in all but one instance, practically no potassium. The potassium intake again became significant, however, upon resumption of oral fluids and food.

During recovery while on either parenteral or oral intake all subjects retained sodium and chloride. The chloride space, assumed at the start to be 15 per cent of the initial body weight for purposes of calculation expanded progressively until, in some instances, it exceeded by a considerable margin the volumes usually assigned to extracellular fluid. Hypokalemia appeared, or usually increased further if already present, in six patients. Low levels developed even though urinary losses were only a fraction of a milliequivalent per day. With potassium administration the urinary excretion increased, but did not equal the intake. This retention points to preexisting deficits and suggests renal conservation of potassium. The data do not identify the renal and extra-renal losses of potassium in the etiology of potassium deficits incurred prior to these studies. They do point, in spite of undue rises in the chloride space employed to partition external balances between cells and extracellular fluid, to a combination of dilution of extracellular fluid and transfer of

potassium into cells as the mechanism of hypokalemia during therapy.

Antifibrillatory Actions of an Alpha-Fagarine Analog (N-Methyl-N-(3-4 dimethoxybenzyl) - B - (4, methoxyphenyl) - Ethylamine - HCL). JOSEPH R. DIPALMA, (by invitation) JOHN E. SCHULTS, RICHARD A. REISS and JOSEPH J. LAMBERT, Brooklyn, N. Y.

The exact chemical structure of α Fagarine is unknown. A new compound, approaching its proposed formula and having similar biological effects and superior in respects to potency with low toxicity has been synthesized.

In cats the new compound is 2.3 times more active than quinidine and 1.3 times more active than α Fagarine in stopping electrically induced auricular fibrillation. The effects on blood pressure, pulse and EKG are similar to quinidine. Nevertheless, its acute i.v. toxicity for cats is less than either quinidine or α Fagarine.

When assayed in humans with normal hearts the Q-T segment is increased on the average by 0.06 sec. with a dose of 100 mgm. and by 0.09 sec. with a dose of 200 mgm. i.m. Similar doses of quinidine in the same patients increased the Q-T segment by 0.01 and 0.03 sec. respectively. This may be taken as good evidence that the drug is capable of increasing the refractory period of heart muscle and that it is more potent than quinidine in this respect.

In 5 patients with chronic auricular fibrillation the f-f interval was increased on the average 28.2 per cent with a dose of 100 mgm. i.m. This slowing of the rate of auricular circus movement is much greater than could be anticipated from a similar dose of quinidine.

Clinical study of 10 patients, 7 with auricular fibrillation, 2 with auricular flutter and 1 with ventricular tachycardia permit the following conclusions. Two cases of auricular fibrillation and one with flutter were restored to a regular sinus rhythm. In one instance restoration occurred with a dose of only 50 mgm. i.m. A very serious disadvantage of the drug is that like α Fagarine it has a tendency to initiate ventricular ectopic foci. Because its chemical structure is known the possibility exists of developing a derivative of equal potency but lacking this undesirable feature.

A Method for Estimating, preoperatively, the Function of Thyroid Tumors; Its Significance in the Diagnosis and Treatment. BROWN M. DOBYNS and BENGT SKANSE (Introduced by Edward D. Churchill), Boston, Mass.

An estimate of function of a discrete thyroid nodule contributes in a major way to the diagnosis and treatment of such a goiter. Many studies indicate that function of such nodules can be related to their avidity for radioiodine. An in vivo technic has been devised for detecting the degree of concentration of radioiodine in various parts of the thyroid. This technic permits an estimation of the relative function of discrete nodules as compared with the remaining thyroid.

The conventional Geiger-Muller counters are incapable of locating point sources within the thyroid because of the anatomical and physical relationships involved.

A specially constructed directional counter has been found capable of locating and differentiating relative concentrations of radioiodine over small areas of the thyroid. In more than 50 patients the results of observations made with this technic were found to agree with radioautographs made from the same tissue removed at operation. Such differentiation in the function of thyroid nodules contributes significantly to the intelligent treatment of these goiters.

Hyperfunctioning adenomas of the thyroid are recognized as a source of clinical or masked hyperthyroidism which may produce cardiac damage. Their recognition and removal are often followed by remarkable improvement. This method of preoperative study has been of value in recognizing many such lesions.

By this method of locating the concentrated radioiodine, an incidental non-functioning nodule in the thyroid of Graves' disease may be distinguished from a hyperfunctioning adenoma producing hyperthyroidism. Since the management of these two problems may differ, the observations are important.

In our experience cancer of the thyroid collects less radioiodine than normal thyroid tissue. The finding of a nodule with a decreased or absent function eliminates the large group of hyperfunctioning nodules and thus increases our suspicion of malignancy.

Pulmonary Circulatory Dynamics in Mitral Stenosis and Left Heart Failure. J. W. DOW (by invitation), L. DEXTER, and (by invitation) F. W. HAYNES, J. L. WHITTENBERGER, and B. G. FERRIS, Boston, Mass.

Pressures in the pulmonary artery and pulmonary "capillaries" were measured with Hamilton and saline manometers. Blood flow through the lung was determined by the direct Fick principle. Pulmonary arteriolar resistance was determined by the Poiseuille equation.

In patients with mitral stenosis and left heart failure, pulmonary "capillary" pressure was elevated. In mild cases, the pulmonary artery pressure was likewise elevated, while the pulmonary artery-pulmonary "capillary" pressure gradient, blood flow, and pulmonary arteriolar resistances remained normal. In more advanced stages, the pulmonary "capillary" pressure at rest exceeded the osmotic pressure of plasma, the pulmonary artery pressure became extremely elevated, and the pulmonary arteriolar resistance rose to high levels due in part to a decreased cardiac output but mainly to a high pulmonary artery-pulmonary "capillary" pressure gradient.

It is concluded that in mitral stenosis and left heart failure (1) the pulmonary "capillary" pressure is elevated, sometimes exceeding the osmotic pressure of plasma, (2) the pulmonary artery pressure rises to a similar extent in early cases and in advanced cases the rise is out of all proportion to the rise in pulmonary "capillary" pressure due to constriction of the pulmonary arterioles thus producing an obstruction to blood flow proximal to the pulmonary capillary bed.

Effect of Treatment on Renal Functions and Electrolyte Excretion in Congestive Heart Failure. DAVID P.

EARLE, JR., (by invitation) SAUL J. FARBER and JOHN D. ALEXANDER, and LUDWIG W. EICHNA, New York, N. Y.

Serial observations on renal functions, daily electrolyte excretions and cardiovascular status were made on 2 patients during various phases of congestive heart failure. They were maintained on regular hospital diet and moderate ward activity, and received no therapy other than digitalis products. Glomerular filtration rate (GF), renal plasma flow (PF) and TmPAH were reduced while the filtration fraction (FF), venous pressure (VP) and weight were elevated in both during the congestive failure phase. As edema was lost there was fall in VP and FF and some improvement in GF and PF. These changes, however, were slight and the functions did not return to normal. Electrolyte excretions paralleled water balance, but at no time did electrolyte excretions fall to very low levels.

The acute effects of intravenous digoxin on the above functions were also studied. Rather prompt increases in water and sodium excretions were noted, even in the absence of significant change in GF, PF or FF. Water and electrolyte excretions appeared to be inversely related to the VP. In instances where VP fell but remained elevated well above normal, little or no increase in water or electrolyte excretion was observed. Similarly, marked diuresis was observed following intravenous injection of aminophyllin, without significant change in renal hemodynamics.

These data are compatible with the thought that impaired water and electrolyte excretion in congestive heart failure is not necessarily related primarily to the commonly observed changes in renal hemodynamics. It appears that increased renal venous pressure may play an important role in retention of water and sodium in congestive heart failure of man. Data bearing on the possibility that cardiac glycosides may act on renal tubular mechanisms of electrolyte reabsorption is also presented.

Effect of Protein Starvation on the Clinical Course, Liver Function, and Liver Histochemistry of Patients with Active Liver Disease. RICHARD ECKHARDT, NORMAN ZAMCHECK, RICHARD SIDMAN and GEORGE GABUZDA (Introduced by Maxwell Finland), Boston, Mass.

To clarify the role of protein in the treatment of human liver disease, 3 deeply jaundiced patients with active alcoholic cirrhosis (fatty) were provided with food adequate in calories but not sufficient in protein to maintain nitrogen equilibrium (0.5, 0.2, and 0.0 grams per kilogram body weight daily for 24, 8, and 11 days, respectively). Although negative nitrogen balance persisted, each manifested clinical improvement, a decrease in liver size and tenderness, a rapid fall in serum bilirubin and in urine bile, and a progressive decrease in urine urobilinogen (measured in 2 patients). Nitrogen balance became positive when 1.0 to 1.4 grams of protein per kilogram daily were given. Improvement continued, but appeared less marked because of the extent of previous change. The serum albumin concentration and thymol turbidity and

flocculation tests, although frequently abnormal, showed no consistent pattern of change.

Histochemical studies were made of liver tissue obtained by biopsy from the last 2 patients before and during the low protein period and during the increased protein feeding period. Five "protein starvation" biopsies showed an initial low cellular protein content which further diminished, moderate to marked fat which remained unaltered, and shrunken cells. Necrosis was not observed. The three "protein repletion" biopsies showed a marked increase in protein content, a progressive decrease in fat, and a return of cell size and appearance toward normal. Liver glycogen progressively increased during both periods of study, while fibrosis (minimal) remained unchanged.

It is concluded that, under the conditions of this study, improvement in clinical status and in liver function occurred in spite of negative nitrogen balance occasioned by diets grossly deficient in or devoid of protein. Liver histology, however, failed to reflect this improvement, and morphologic improvement appeared only with the provision of adequate exogenous protein and the maintenance of positive nitrogen balance.

Studies on the Pathogenesis of Nephrotic Edema. HOWARD A. EDER and FRANCIS P. CHINARD (by invitation), HENRY D. LAUSON, and (by invitation) ROGER L. GREIF, ALMA HILLER, GEORGE C. COTZIAS and DONALD D. VAN SLYKE, New York, N. Y.

The availability of concentrated human albumin for intravenous administration has made possible extensive studies of the sequence of events occurring during the development of nephrotic edema. The initial event is proteinuria. The renal mechanism involved has been examined in nephrotic patients by measurements of the albumin clearance under conditions of changing plasma concentrations following infusion of albumin. It has been found that the excretion is proportional to the plasma concentration, and that the albumin/creatinine clearance ratio is constant over a wide range of concentrations, unless the blood volume increases, in which case the ratio also increases. Our present concept is that in nephrosis albumin is filtered through the glomerular membrane in abnormally large amounts, and that almost all of the filtered albumin is excreted, tubular reabsorption being relatively slight. The continued excretion of albumin results in hypoalbuminemia, and, in accordance with the Starling hypothesis, the plasma volume decreases absolutely as well as relative to the extracellular fluid volume. The diminished plasma volume is believed to be responsible for the decreased renal excretion of water and salt which is the immediate cause of edema. This belief is based on the observation that the excretion of these substances rises sharply when the plasma volume is expanded following the administration of albumin. The fact that the rate of glomerular filtration is increased at the same time suggests that the retention of salt and water during accumulation of edema may be attributed at least in part to a decrease in the rate of glomerular filtration, relative to the intrinsic (i.e., non-hormonal) tubular reabsorptive activ-

ity with respect to water and salt. In addition, there is some evidence to suggest that increased secretion of salt-active adrenal hormones and antidiuretic hormone may play a role in the retention of salt and water.

The Mechanism of Anemia and Its Influence on Renal Function in Chronic Uremia. CHARLES P. EMERSON, JR., and BELTON A. BURROWS (Introduced by Chester S. Keefer), Boston, Mass.

The assumption that anemia in uremic patients is due to inadequate blood production rather than to excessive blood destruction has been tested in four cases of chronic nephritis with marked anemia and azotemia. Hematologic and metabolic studies were performed before and after successive transfusions of marked red cells which in each instance could be distinguished by means of selective agglutination technics from those of the recipient. The relative rates of erythropoiesis and hemolysis were estimated from the results of serial hematologic examinations, including separate measurements of the concentration and total volume of circulating donor and recipient red cells, the degree of reticulocytosis and of bilirubinemia. Parallel determinations were made of the concentrations of non-protein nitrogen and electrolyte constituents of the blood, the total volume of extracellular fluid, and the volume and composition of the urine, as a basis for evaluating the influence of anemia and the effect of corrective blood replacement on renal function.

The data obtained indicated that anemia in these cases was mainly attributable to a depression of erythropoiesis, but also suggested that a contributory factor in its pathogenesis was the presence of excessive hemolytic activity, evidenced by the unusual rapidity with which donor red cells were destroyed following transfusion, their elimination from the blood of the recipient occurring at 1.5 to 3 times the normally expected rate. With respect to the clinical and metabolic effects of massive transfusions it was impossible, in this series of patients, to demonstrate any correlation between the degree of renal decompensation and the grade of coexisting anemia. Complete correction of the latter by means of artificial replacement therapy failed in all instances to lessen or to increase the severity of the uremic manifestations or to influence the progress of the underlying renal disease.

The Insulin Glucose Tolerance Test—An Improved Procedure for the Detection of Hypoglycemia Unresponsiveness in Pituitary and Adrenal Insufficiency. FRANK L. ENGEL and (by invitation) JAMES L. SCOTT, Durham, N. C.

The usefulness of the insulin tolerance test for the diagnosis of conditions characterized by hypoglycemia unresponsiveness is limited by the danger of serious hypoglycemic reactions in those patients in whom the test should be most valuable. Smaller doses of insulin often yield equivocal results requiring repetition of the test. To overcome these objections advantage has been taken of the fact that hypoadrenal and hypopituitary patients not only exhibit hypoglycemia unresponsiveness after insulin but also a de-

layed response to oral glucose during hypoglycemia. By giving 0.8 gm. of glucose per kilogram orally to the fasted subject 30 minutes after 0.1 unit insulin per kilogram intravenously, hypoglycemic manifestations are aborted but hypoglycemia unresponsiveness is still apparent. Blood sugars are determined before insulin and at 30, 60, 90, 120, and 180 minutes. The results are expressed as per cent of the fasting blood sugar. In 9 normal individuals and 9 patients with non-endocrine diseases the mean maximal rises in blood sugar after glucose were 149 and 128 per cent of the initial blood sugar at 90 minutes with a return to the fasting level at 180 minutes. In 7 observations on Addison's disease and 6 on panhypopituitarism the mean maximal rises were only 82 and 84 per cent of the initial blood sugar respectively and occurred at 180 minutes. None of the latter patients achieved a blood sugar after insulin and glucose as high as the lowest peak in the two control groups. As a group, 6 patients with anorexia nervosa had a mean response not significantly different from the controls. However, one patient fell clearly into the hyporesponsive range and one was borderline. Three patients with primary myxedema showed normal curves. Neither pretreatment with lipoadrenal cortex, intravenous cortical extract (50 ml.) or epinephrine before and after insulin restored the response of the hyporesponsive patients to normal.

Skin Reactions to Toxoplasma Antigen in People without Known History of Infection. HARRY A. FELDMAN and ALBERT B. SABIN, Cincinnati, Ohio.

One hundred and forty-two residents of the Cincinnati area ranging in age from 3 months to 81 years were surveyed for their reaction to the intracutaneous injection of toxoplasma antigen prepared from infected chorioallantoic membranes of chicken eggs. The positive reactions, consisting of red, indurated, swollen, tender and itching lesions ranging in size from 60 × 50 mm. to 10 × 10 mm. (the latter being relatively rare), reached their maximum in 30 to 48 hours. The incidence of positive reactions among at least 20 individuals in each age group listed was as follows: 0-4 years—0; 5-9 years—5%; 10-19 years—14%; 20-29 years—20%; 30-39 years—50%; 40-49 years—65%; 50-81 years—65%. A positive skin test was invariably associated with demonstrable toxoplasma antibody in the serum. Toxoplasma antibody in varying titer was also found in the serum of 7 of 18 persons (39%) with negative skin tests. Further evidence in favor of the high incidence of toxoplasma antibody among the "normal" adult population was obtained when a 15-20 fold concentrated, commercial preparation of gamma globulin prepared from the plasma of more than 20,000 American adults was found to contain toxoplasma antibody in a titer of 1:256 as determined by the dye test. No agent having an antigen in common with toxoplasma and capable of giving rise to antibodies for this protozoon parasite has been found thus far. Therefore, one must consider the possibility that inapparent infection with toxoplasma may be very widespread, although it is rather curious that the incidence of antibodies (and presumably infection) should rise so sharply after the 20th year of life. The toxoplasma

skin test is not useful for diagnosis because a strongly positive test bears no relationship to titer of antibody and a negative test is encountered too often in the presence of antibody.

Studies on the Isolation and Identification of Beta-Hemolytic Streptococci from Throat Swabs. A. E. FELLER, (by invitation) GEORGE F. BADGER, JOHN H. DINGLE, (by invitation) RICHARD G. HODGES, and CHARLES H. RAMMELKAMP, Cleveland, Ohio.

Investigations of the role of β -hemolytic streptococci in respiratory disease require (1) sensitive methods for detecting the organisms on throat swabs, especially when they are present in small numbers, and (2) reliable methods for characterizing the organisms so isolated. For the latter purpose, classification according to the serological groups of Lancefield is essential.

The purposes of the present study were (1) to compare the sensitivity of the conventional streak plate method with the technique devised by Pike employing selective media for the detection of β -hemolytic streptococci on throat swabs, (2) to devise a simple method for predicting that a certain organism can be grouped serologically, short of making an extract of each organism and testing it against group specific sera, and (3) to determine the reliability of the rather simple enzyme technique devised by Maxted for preparing extracts of the organisms for serological identification.

Comparison of the streak plate method with the Pike technique showed that 96 per cent of 327 consecutive isolations of groupable streptococci were accomplished by the Pike method; in only 4% of the swabs was the streak plate positive when the Pike plate was negative. After evaluating many procedures it was found that β -hemolysis of sheep blood agar (surface or deep colonies) is a simple and reliable criterion for determining that a streptococcus can be grouped serologically (Lancefield). Organisms failing to produce β -hemolysis on sheep blood agar have thus far not been groupable. Comparison of the enzyme technique of Maxted with the conventional formamide method of preparing extracts of streptococci for serological grouping has demonstrated essentially complete agreement when employing streptococci producing β -hemolysis on sheep blood agar. The Maxted technique is extremely simple and the extracts react with specific sera in 15 to 30 minutes.

Iron Metabolism in Hemochromatosis. C. A. FINCH (Introduced by James P. O'Hare), Boston, Mass.

Observations have been made relating to the diagnosis, pathogenesis, and treatment of hemochromatosis.

Iron excess may be recognized by sternal puncture, by the per cent utilization of radioiron for hemoglobin production, and by determining the per cent saturation of the iron binding protein of the serum. The latter has proved particularly useful in the recognition of hemochromatosis.

Since little iron is excreted in man, hemochromatosis must be associated with increased iron absorption. In animals it has been possible by dietary means to produce excessive iron absorption, indicating that the normal mu-

cosal regulation of iron absorption is not effective in certain circumstances. It is also suggested by similarity in iron distribution in these experimental animals, in patients with multiple transfusions and in idiopathic hemochromatosis, that the iron in all instances represents storage iron. Accordingly several patients have been repeatedly phlebotomized. In contrast to normal subjects who rapidly exhaust their iron reserves and develop anemia, patients with hemochromatosis are able to give up large amounts of iron without evidence of iron deficiency.

These studies provide methods of identifying enlarged stores of iron at an early stage in the clinical course of hemochromatosis and by phlebotomy a means of removing the excessive iron found in this disease.

Sweat Sodium Concentration in Thyrotoxicosis. FRANCIS W. FITZHUGH and ARTHUR J. MOSELEY (by invitation), and ARTHUR J. MERRILL, Atlanta, Ga.

Conn has demonstrated an inverse relationship between the sweat sodium concentration and the activity of the sodium-retaining factor of the adrenal cortex. In the course of other studies, we found a low sweat sodium concentration in 10 of 11 patients with thyrotoxicosis. Our average for 26 determinations in 18 normal subjects was 49.1 mEq./l. with a range of 15 to 92 mEq./l., all but 4 being between 24 and 70. The average for 10 of 11 patients with thyrotoxicosis was 14.2 mEq./l. with a range of 2.4 to 26.6. The remaining patient had a sweat sodium concentration of 46.2 mEq./l. The BMR's of these patients ranged from +42 to +80.

Five normal individuals were placed on thyroid extract, 2 grams daily. Three of these had a drop in sweat sodium concentration from 51 to 31, 61 to 32.5, and 92 to 22 on the 7th, 7th, and 4th days respectively. One subject had a low control level of 19 mEq./l. and his sodium concentration rose to 25 on the 7th day. The fifth individual showed an increase from 32 to 63 mEq./l. on the 10th day. Two of those who had a fall in sweat sodium concentration were restudied with daily sweat sodium determinations. One of these had a marked diminution on the 9th day from 38 to 19.5 followed by a rebound to 42 on the 11th day. The other showed no significant change. Reciprocal changes occurred in the sweat potassium concentration. No change was noted in the number of circulating eosinophils or in the BMR.

The determination has been of assistance in diagnosis in two patients with thyrotoxicosis whose BMR's were unsatisfactory and in whom the diagnosis was uncertain.

In summary, a new method is offered for the diagnosis of thyrotoxicosis which shows little overlapping with the normal.

Metabolic Studies on Cushing's Syndrome. P. H. FORSHAM and E. FLINK (by invitation), K. EMERSON, JR. and G. W. THORN, Boston, Mass.

Eosinopenia (less than 50 cells per cu. mm.) appears to be characteristic of Cushing's syndrome and an elevated urinary uric acid: creatinine ratio (greater than 0.8) frequently occurs. Such changes have been reproduced in normal subjects given adrenocorticotrophic hormone and

in patients with Addison's disease on Compounds E and F. Following successful treatment, these values return to normal and their change constitutes a helpful indicator of the effectiveness of therapy.

The refractoriness of certain patients with Cushing's syndrome to the salt retaining action of desoxycorticosterone (Soffer) has been confirmed. In a study on the metabolic effects of Compound E in patients with Addison's disease it was found that 100 mg. of Compound E daily led to slight sodium and chloride retention. In contrast, such patients when treated with desoxycorticosterone and showing marked sodium retention exhibited a paradoxical increase in sodium and chloride excretion when given additional Compound E. On the basis of these observations on the competitive renal action of desoxycorticosterone (a powerful salt retainer) and Compound E (a weak salt retainer), it is suggested that the desoxycorticosterone acetate fastness of certain patients with Cushing's syndrome may be due to the blocking action on the renal tubule of an excess of adrenal steroids of the 11,17-oxytype.

Electrolyte Abnormalities in Chronic Congestive Heart Failure; Effects of Administration of Potassium and Sodium Salts. CHARLES L. FOX, JR., CHARLES K. FRIEDBURG and ABRAHAM G. WHITE (Introduced by A. R. Dochez), New York, N. Y.

Plasma, edema fluid and urine electrolytes were studied during congestive failure. Plasma sodium was significantly below normal in twenty-seven of thirty patients. Plasma chlorides and potassiums ranged from below to above normal; chlorides, however, were relatively increased. Urinary sodium was low whereas potassium was high. Repeat measurements after recovery in five patients showed that the low sodiums had increased, whereas chloride and potassium shifted toward normal.

During mercurial diuresis sodium chloride excretion increased but sodium in urine was usually hypotonic; chlorides however equalled or exceeded sodium. Potassium excretion increased fivefold.

The data indicated that failure is frequently associated with hypotonic extracellular fluid excessive in chloride and that during recovery, relatively more water, chloride and potassium are excreted than sodium. The possibility that some of the water excreted was of intracellular origin is also supported by the rise in the non-colloidal extracellular osmotic pressure which might draw water from cells.

Accordingly, sodium lactate was given to raise extracellular sodium and bicarbonate concentrations, and potassium acetate to raise intracellular potassium and bicarbonate. Up to 400 mEq. of each were administered orally for periods up to thirty days to six patients in failure. Mercurial diuretics previously required were omitted. Loss or no gain in weight occurred despite positive balances of sodium and potassium. Sodium excretion increased markedly although subnormal plasma sodium initially decreased further. Shift of water from intra- to extracellular compartments is thereby suggested.

Individual administration of the chloride and acetate of Na or K increased weight two to six kilograms therefore

mercurials were required after each single salt period. Simultaneous administration of sodium chloride and potassium acetate caused minimal weight gain despite positive Na, Cl, and K balances.

Extracellular hypotonicity may be associated with intracellular hypotonicity and depletion, hence separate readjustment of either compartment augments edema. Intracellular abnormalities may also be important in failure.

Quantitative Studies on Pulmonary Absorption of Aerosols Made with Solutions of Sodium p-Amino Hippurate. WILLIAM FRANKLIN and CLARENCE DENTON (by invitation), and FRANCIS C. LOWELL, Boston, Mass.

In the course of laboratory studies on the effect of aerosolized drugs and allergenic extracts in pulmonary disease, the need arose for a method of determining, with some degree of accuracy, the amount of material retained by the subject. The use of penicillin (blood levels) or radioactive materials (tissue concentrations) was considered and discarded as poorly adapted to the problem. Promising results have been obtained with aerosolized solutions of sodium p-amino hippurate. This compound is well tolerated and is rapidly absorbed and quantitatively excreted in the urine. The procedure followed was (1) to expose the subject to a measured volume of a standard aerosol of PAH, (2) to collect material not retained by the subject, and (3) to collect the urine over a six hour period, with determination of the amount of PAH after hydrolysis with acid.

The aerosol, produced by nebulizers receiving oxygen delivered at an accurately measured rate, was passed over concentrated sulfuric acid in order to increase stability by partial drying into a spirometer (which provided the flexibility required by the intermittent removal of the aerosol during the subject's respiration) and through a flutter valve to the subject. Expired aerosol passed through a second flutter valve and was collected in a highly efficient impinger. Measurements were made of the PAH delivered, the PAH in the collecting impinger, the valve assembly, and the urine.

Repeated tests have shown that agreement within 10 per cent was obtainable between (1) the amount delivered and (2) the sum of that found in the urine, the expired air and valve assembly. The following results were obtained in one test with subject inhaling aerosol for ten minutes: amount of PAH delivered—96 mgms.; amount in expired air and valve assembly—54 mgms.; amount in urine—42 mgms.

Studies with a New Method for Quantitative Measurement of I^{131} Content of the Thyroid Gland in Man. A. STONE FREEDBERG, ALVIN URELES and MARVIN VAN DILLA (Introduced by H. L. Blumgart), Boston, Mass.

Direct measurement of uptake of radioactive iodine by the thyroid has been attempted by two techniques of external counting: (1) a Geiger-Mueller counter is placed against the neck over the thyroid gland; (2) the tube is at varying distances from the neck. In both instances, the variation in thyroid size and location (depth geometry

and absorption) have not permitted quantitative measurement of I^{131} content.

With 4-6 platinum cathode Geiger-Mueller counters connected in parallel and arranged in a circle 36-120 cm. in diameter, a central source of radiation was moved about within a sphere 8-32 cm. diameter concentric with the circle of counters; the maximum variation in counting rate was 5 per cent. When the source was surrounded by muscle, bone, water and saline in quantities approximating the human neck there was no significant change in counting rate. With a constant amount of radioactivity in volumes varying from 5-350 cc. in different shaped containers no significant change in counting rate was observed. Identical amounts of I^{131} in 5 cc. and in 2,000 cc. of water placed in gallon jugs showed a decrease of 5 per cent in the counting rate in the latter instance.

Preliminary studies in patients with varying thyroid gland size are consistent with mock up experiments. With a 4 tube circle of 104 cm. diameter, a central "sphere" is present approximately 20 cm. in diameter within which movement of the head and neck gave no significant change in counting rate. Thyroid uptake and urinary excretion has been measured by this method in euthyroid subjects and in patients with various thyroid disorders and will be reported.

It is concluded that quantitative measurement of I^{131} content of the thyroid gland in man is obtained by the above described method independent of the size and location of the gland.

Evaluation of the Dye Method for Estimating Blood Volume in Man. EDWARD D. FREIS, JOSEPH R. STANTON and CHARLES P. EMERSON (Introduced by William L. Fleming), Boston, Mass.

The higher estimation of blood volume using T-1824 as contrasted with labeled red cells has cast doubt on the validity of dye methods for determining blood volume. In man the mixing time of T-1824 was relatively complete within the first three minutes following which there was a uniform and therefore detectable disappearance of circulating dye which could be corrected by extrapolation. Repeated injections of T-1824 at short intervals indicated that there was no undetected dye loss during the initial mixing period. Simultaneous determinations of arterial and hepatic venous concentrations of dye failed to demonstrate significant escape of T-1824 in the liver.

Measurement of the relative velocities of dyed plasma and identified red cells during a single circulation through a peripheral vascular bed revealed that in the capillaries the mean velocity of the cells was greater than that of plasma. The capillary hematocrit, therefore, must be lower than the large vessel hematocrit. These observations support the validity of the dye method for measuring plasma volume and indicate that the high values obtained for total blood volume with T-1824 are not due to undetected escape of dye from the circulation but to the discrepancy in the proportion of red cells and plasma in large versus small vessels.

A Quantitative Study of the Rate of Renal Excretion of Digitoxin. MEYER FRIEDMAN and (by invitation) RENE BINE, JR. and SANFORD O. BYERS, San Francisco, Calif.

Hitherto, it has not been possible to determine with quantitative methods the role of the kidney in the excretion of digitalis glycosides. However, by means of the embryonic duck heart method, as little as one microgram of digitoxin could be detected in the 24 hour urinary output of the rat. This technique therefore enabled us to study the rate of renal excretion of digitoxin in this animal after its parenteral administration.

A series of 10 rats were given by intramuscular injection 0.1 mcg. of digitoxin per gram of body weight (or approximately 15 mcg. per rat) and the urine was collected daily for three days. Each 24 hour urine sample was analyzed for its digitoxin content. No digitoxin could be detected in the initial 24 hour urine collection of any rat, indicating that less than one microgram of digitoxin had been excreted during this period. Likewise no digitoxin was found in the 48 or 72 hour urine sample of any rat.

A series of 12 rats therefore were injected with 1.0 mcg. of digitoxin per gram of body weight (approximately 225 mcg. per rat). It was found that there was an average excretion of 6.0 mcg. of digitoxin (2.5 per cent of amount injected) during the first 24 hours following injection. No digitoxin, however, was detected in any of the urine samples collected during the 48 or 72 hours following injection.

The above results indicate that the renal excretion of digitoxin in the rat is either nil or relatively insignificant even after the administration of relatively huge amounts of the drug.

Effect of Lipotropic Agents, Testosterone Propionate, and Dietary Protein upon Nitrogen Balance in Patients with Liver Disease. GEORGE J. GABUZDA and RICHARD D. ECKHARDT (by invitation) and CHARLES S. DAVIDSON, Boston, Mass.

Four patients with Laënnec's cirrhosis and one with infectious hepatitis were maintained on basal diets providing 25, 50, 75, 100 and 100 grams of protein daily. Initial control nitrogen balances averaged -4.2, +1.6, +3.3, +5.4, and +6.8 grams daily, respectively. The effect of supplements of choline and methionine, of testosterone, and of dietary protein upon these balances was determined.

The oral administration of 4.5 to 9.0 grams each of choline chloride and dl-methionine simultaneously daily for 6 to 9 days to these 5 patients did not have a nitrogen sparing effect above that which could be accounted for by retention of part of the nitrogen contained in these agents.

Subsequent to control periods following the administration of choline and methionine, 25 mgm. of testosterone propionate was administered intramuscularly daily for 6 days to the 3 patients with intakes of 50, 100, and 100 grams of protein daily. Their ability to respond to a protein anabolic stimulus was indicated by increased nitrogen retentions of 1.2, 2.6, and 1.4 grams daily.

After the reestablishment of nitrogen balances comparable to initial control periods, increases in dietary protein of 50 grams daily in the 3 patients on basal intakes of 50, 75, and 100 grams of protein were accompanied by increases in daily nitrogen retention averaging 2.6, 3.8, and 7.1 grams, respectively. The patient in negative nitrogen balance while receiving a 25 gram protein basal diet promptly established nitrogen equilibrium when the protein intake was increased to 50 grams, although previously choline and methionine had failed to spare nitrogen.

Although testosterone propionate had a protein anabolic effect in these patients with liver disease, it is concluded that positive nitrogen balance was most effectively achieved by adequate dietary protein. No appreciable nitrogen sparing action could be attributed to choline and methionine in the quantities used.

The Effect of Anoxic Anoxia on the Human Kidney. MORTON GALDSTON, EUGENE Y. BERGER, and SEYMOUR A. HOROWITZ (Introduced by J. Murray Steele), New York, N. Y.

Several factors, among them anoxia, may play a part in retention of salt and water in heart failure. The following experiments were designed to test the effect of renal anoxia per se on these phenomena. In 7 subjects with normal cardiovascular, respiratory systems, renal function during inhalation of 14 per cent oxygen (arterial oxygen tension of approximately 50 mm. Hg), a degree of anoxia which results in barely perceptible changes in circulatory and respiratory dynamics, was compared with renal function during the preceding and subsequent periods breathing room air. Similar studies were conducted in 2 emphysematous subjects with arterial blood oxygen tensions of approximately 50 mm. Hg while breathing room air and again, while breathing 100 per cent oxygen. Periods breathing room air were considered as the anoxic periods. Observations on respiration and circulation were also made.

Renal plasma flow remained the same or tended to increase during anoxia in all normal subjects. In the emphysematous subjects it decreased during inhalation of 100 per cent oxygen. Change in filtration rate during anoxia was not consistent in direction or degree. The most pronounced effect of anoxia on renal function was an invariable and frequently marked increase in the rate of excretion of sodium, chloride, and water. The alterations in respiration and circulation which occurred during inhalation of 14 per cent and 100 per cent oxygen do not seem to account for the changes in renal plasma flow. The conclusion is drawn that anoxic anoxia alone does not appear to result in reduction of renal plasma flow or retention of salt and water. Other electrolyte changes studied will be presented.

Transfer to Normal Red Cells of an Agglutinin Demonstrable in the Acidified Sera of Patients with Acquired Hemolytic Jaundice. FRANK H. GARNER (Introduced by Thomas Hale Ham), Boston, Mass.

Studies of defibrinated blood samples from 15 patients with acquired hemolytic jaundice have shown agglutina-

tions of N_{15} were found in the uro- and coproporphyrin isolated during different periods. The data will be discussed in relation to hemoglobin metabolism.

The Neuromuscular and Ganglionic Blocking Action of bis-Trimethylammonium Decane and Pentane Dibromide (C 10 and C 5). DAVID GROB and DUNCAN A. HOLADAY (by invitation) and A. McGEHEE HARVEY, Baltimore, Md.

Paton and Zaimis studied the effects in animals of polyethylene bis-trimethylammonium salts of the general formula $(CH_3)_3N-(CH_2)_n-N(CH_3)_3(Br)_2$. They found the C 10 derivative ($n=10$) to be a neuromuscular blocking agent and the C 5 derivative ($n=5$) an autonomic ganglionic blocking agent. The administration of these compounds to man has shown that they are very effective in their blocking action at these sites and are clinically useful. The intravenous injection of 2.5 mg. of C 10 in normal subjects resulted in almost complete paralysis of the muscles of the extremities, with relatively slight weakness of swallowing, speech, and respiration. C 10 was administered to more than 60 patients for the relaxation of skeletal muscles during convulsive shock therapy and surgical anaesthesia. The effective dose of C 10 was one-third that of d-tubocurarine on a weight or molar basis, and it had relatively less effect on the bulbar and respiratory muscles. The action of C 10 was of shorter duration than that of d-tubocurarine. C 10 caused a progressive decline in the muscle action potential in response to nerve stimuli. In contrast to d-tubocurarine it produced no histamine-like effects. C 5 produced evidence of autonomic ganglionic block in doses one-tenth to one-twentieth that of tetraethylammonium, on a weight or molar basis. The intravenous administration of 5 to 20 mg. of C 5 resulted in marked postural hypotension in most normal and hypertensive subjects, and a prompt fall in blood pressure in some hypertensive subjects. These effects persisted for as long as 3 to 6 hours.

The Role of the Kidney in the Pathogenesis of Malignant Hypertension as Indicated by a Study of Dogs Maintained with an Artificial Kidney after Nephrectomy, Implantation of the Ureters into the Bowel or Vena Cava, and Ligation of the Ureters. ARTHUR GROLLMAN, E. E. MUIRHEAD and JOHN VANATTA (Introduced by Gladys Fashena), Dallas, Texas.

That the kidney plays a role in the animal economy in addition to its recognized function as an excretory organ has been inferred, particularly in relation to hypertension. However, direct experimental evidence bearing on this problem has not been possible since nephrectomy or abolition by other means of renal function leads to death from excretory insufficiency before other evidences of renal dysfunction are manifested. The present study is based on observations of more than 150 dogs subjected to (1) bilateral nephrectomy, (2) bilateral ligation or severance of the ureters, or (3) implantation of one ureter into the small bowel or into the vena cava with subsequent removal of the contralateral kidney. Survival of the animals was extended by application of an artificial kidney, the use of

which shall be described. Nephrectomy results in an elevation of blood pressure and pathological changes in the kidneys characteristic of malignant hypertension. These observations, which are contrary to those generally held on the basis of short survival studies, offer direct evidence against the view that renin or other pressor substances play a role in the pathogenesis of hypertension.

In the presence of renal tissue as when a ureter is implanted into the duodenum or vena cava and the contralateral kidney removed, no elevation in blood pressure ensues. However, pathological changes induced by the accumulation of catabolic waste products from the organism are induced. The experiments permit certain conclusions as to the relation of the presence of renal tissue, blood pressure changes and uremia in the pathogenesis of the structural changes observed in malignant hypertension.

The Application of Electron Microscopy to the Study of Connective Tissue Constituents. JEROME GROSS (Introduced by Walter Bauer), Boston, Mass.

The electron microscope because of its high resolving power is capable of revealing the form of aggregation of macromolecules in certain biological systems. When this organization is of a constant and regular pattern one is provided with an accurate fingerprint of the substance which can be used for identification and study of formation and alteration.

The fibrous components of the connective tissue, collagen and elastin, are particularly suitable subjects. Up to the present only normal tissues have been studied, in part for the purpose of providing a base line for the eventual examination of pathological material. An extensive study of collagen from many tissues of many animals has revealed a characteristic fibrillar unit with an axial repeating period of 640 A units and a distinctive pattern of intra-period fine structure.

A study of the elastic tissue of the mammalian aorta, ligamentum nuchae and fish swim bladder has revealed characteristic non-banded branching fibers which when digested by trypsin give rise to long filaments 120A in width in the form of tight, evenly coiled helices produced by the interlacing of finer threads approximately 70A in width. The pitch of the helix, approximately 530A was the same for filaments from human, rabbit and rat aorta as well as ligamentum nuchae. Boiling the tissue in dilute acid to remove collagen prior to enzyme digestion did not affect the filaments.

The influence of pH and temperature on the state of aggregation and helical morphology of these structures will be discussed.

It is concluded that the elastic fiber is a two component system composed of bundles of trypsin-resistant filaments of characteristic form and size imbedded in a trypsin-sensitive, heat-resistant "amorphous" binding matrix. The possible relationship of the elastic properties of the tissue to this structure will also be discussed.

The Effects of Sympathectomy and Other Sympatholytic Procedures on the Cerebral Circulation in Essential Hypertension. JOSEPH H. HAFKENSCHIEL and

HENRY A. SHENKIN (by invitation), SEYMOUR S. KETY and WILLIAM A. JEFFERS, Philadelphia, Pa.

Quantitative data on blood flow, oxygen consumption and vascular resistance of the brain were obtained in hypertensive patients before and after bilateral lumbodorsal sympathectomy (9 patients), differential spinal sympathetic block (17 patients), parenterally administered dihydroergocornine (7 patients) and bilateral stellate ganglion block (5 patients).

Cerebral vascular resistance was significantly decreased following thoracolumbar sympathectomy, differential spinal block and intramuscular dihydroergocornine. Cerebral vascular resistance was not lowered after block of the stellate ganglia bilaterally.

These findings are interpreted as indicating that the increased resistance in the brain of essential hypertension is partially reversible. No evidence has been adduced that this resistance is mediated through the stellate ganglia, but our data do not rule out completely a neurogenic mechanism. It is of interest that the cerebral blood flow and cerebral oxygen metabolism are unchanged with the hypotension of sympathectomy and dihydroergocornine; the cerebral blood flow decreased significantly only when the blood pressure was acutely and severely reduced by differential spinal block.

It is concluded that procedures which produce a prolonged but moderate fall in arterial blood pressure in hypertensives do not bring about a sustained reduction of the cerebral blood flow when measured in the supine position since the vessels of the brain are capable of compensatory dilatation. When the reduction in blood pressure is sudden and excessive, however, the compensation may be incomplete.

Analysis of Mechanism of Cooley's Anemia by Transfusion Survival of Erythrocytes. HENRY E. HAMILTON and RAYMOND E. SHEETS (Introduced by Elmer L. DeGowin), Iowa City, Iowa.

The survival of normal erythrocytes and those from patients with Cooley's trait were studied after transfusion into normal subjects, persons with Cooley's trait, and patients with Cooley's anemia by the Denstedt modification of the Ashby differential agglutination technique. A new method of analysis was employed in which the ratio of the concentrations of the donor's cells to those of the recipient was used to indicate fluctuations in the plasma volume of the recipient, the relative rate of disappearance of transfused erythrocytes, the abnormal destruction of the recipient's cells, and temporary erythropoietic arrest.

It was found that normal erythrocytes normally survive transfusion into subjects with Cooley's trait and Cooley's anemia. The red blood cells from persons with Cooley's trait persist normally in the circulation of healthy subjects and in subjects with Cooley's trait. They also survive a normal span in patients with Cooley's anemia. Analysis of the donor-recipient-cell ratio demonstrates that the mechanism of anemia in Cooley's disease is the rapid destruction of the patient's own erythrocytes. Similar hemolytic episodes of milder degree were also noted in persons

with Cooley's trait. In addition, periods of diminished and augmented rates of erythropoiesis were noted in normal subjects, persons with Cooley's trait, and patients with Cooley's anemia.

(The data will be presented from twelve blood transfusions in which the method was applied in the study of Cooley's anemia. Controls are derived from many other transfusions studied in normal subjects and patients with various blood dyscrasias.)

Effect of Influenza Viral Infection on Ciliary Movements in the Mouse. CARL G. HARFORD and (by invitation) MARY HARA, St. Louis, Mo.

Cells of respiratory epithelium of man and other animals are equipped with cilia and the movements of these hair-like structures are thought to be an important means of natural resistance to bacterial infection. One theory concerning inception of respiratory infection is that viruses destroy the cilia-bearing cells of the epithelium thereby enabling bacteria to gain a foothold. In previous experiments, it was found that lowered pulmonary resistance of the mouse to inhaled pneumococci is associated with the lesion due to infection with influenza virus. The purpose of the present study was to determine whether viral impairment of ciliary action in the bronchi contributes to this lowered resistance since influenza virus is known to attack the bronchial epithelium.

In addition to special stains for demonstration of cilia, their movement was visualized directly in thin slices of fresh lung which were mounted as wet preparations in saline. When the margins of bronchi were viewed under the microscope with reduced light or by phase microscopy, the rapid ciliary beat was seen clearly.

In the viral lesion of 5 days duration, it was found that regeneration of bronchial epithelial cells had taken place and the cilia appeared normal both in morphology and motility. Therefore, in order to observe the maximal effect of viral infection on ciliary action, many lethal doses of virus were employed and observations were made after 2 to 3 days so that the greatest number of epithelial cells would be in the stage of necrosis. Even under these conditions, the majority of ciliated cells were not involved and profuse ciliary movement was observed regularly.

These findings do not support the theory that viruses affect resistance by interference with ciliary action.

Liver and Kidney Function in Rocky Mountain Spotted Fever. GEORGE T. HARRELL, Winston-Salem, N. C.

Chemotherapy or specific immunotherapy is administered to eradicate an etiologic agent. Specific therapy, as opposed to supportive therapy, may not prevent or control physiologic disturbances induced by an infection. From 1942-1947, liver and kidney function tests were performed on 16 children and 10 adults with Rocky Mountain spotted fever. Tests were done on admission, at the clinical peak of the disease, during convalescence and after discharge.

Livers: Less than 3 gm. of galactose was excreted in 11 tests. In 21 tests, less than 27 gm. (oral) or 67 gm. (IV) of hippuric acid was excreted in 13 instances.

Bromsulfalein was retained 6 times in 18 tests. Serum bilirubin exceeded 4 mg. per cent only once. Prothrombin time was elevated markedly in 2 patients. Serum albumin was below 3.5 gm. per cent in 18 patients.

Kidney: Urea clearance was below 70 per cent in 9 of 13 patients. Phenolsulfonphthalein excretion exceeded 65 per cent (50 per cent in children) in 9 patients. The NPN exceeded 40 mg. per cent in 7 patients. Slight albuminuria was occasionally observed. RBC were found in the urine in 5 patients and casts in 8.

Liver damage, related in degree to the clinical severity of the disease, was greatest by hippuric acid test and occurred with or following the clinical peak. BSP, prothrombin, and albumin alterations occurred during the acute febrile phase. Function returned to normal with convalescence.

Kidney damage was less marked in degree, unrelated to the clinical severity, occurred before or during the clinical peak and returned to normal with convalescence. No glomerulonephritis was seen. Azotemia is probably due to fluid and circulatory disturbances rather than renal damage.

In 18 patients a high protein diet protected against changes in serum albumin and hippuric acid excretion; it did not decrease renal function but occasionally overloaded the kidney.

The mechanism responsible for the changes is obscure.

The Response to Adrenocorticotrophic Hormone in Patients with Scleroderma and the Therapeutic Use of Testosterone. SAUL HERTZ and (by invitation) PETER H. FORSHAM, Boston, Mass.

Scattered evidence has pointed to an endocrine factor in scleroderma, but none put forth to date incriminates any specific glandular defect. Suggestive features of a positive type include the marked incidence of hypercreatinuria, reduced creatine tolerance tests, low urinary 17-ketosteroid excretion and a relationship of onset of periods of exacerbation to menstruation, menopause, etc. Predominance of the disease in the female sex, and in prepubertal males, has been impressive. Disorders of calcium metabolism (ectopic calcification), negative nitrogen balance and marked wasting have been emphasized. We have confirmed these findings in our series. The creatinuria, myopathy, negative nitrogen balance, pigmentation and occasional low serum sodium and chloride levels are compatible with some degree of gonadal or adrenal hypofunction. That this might be secondary to pituitary underactivity is suggested by low FSH titres, I^{131} uptake by the thyroid and 17-ketosteroid excretion.

Seven cases have been studied from the standpoint of 17-ketosteroid excretion, creatine tolerance and responses to epinephrine and ACTH.

17-Ketosteroid values on 24-hour urines in this group ranged from 1.2 mg. to 6.0. However, 48-hour tests with ACTH (40 mg. per day) led to a marked rise in 17-ketosteroid excretion and other evidences of "S" factor activation.

Since ACTH is not available for therapeutic application, we chose to observe 4 patients on high dosage of tes-

tosterone propionate intramuscularly. Dosage ranged from 25 to 50 mgs. \times 3 per week. Gradual disappearance of skin lesions, as well as improvement in esophageal involvement (radiologic evidence) took place. Rapid weight gain and increased appetite and strength together with the establishment of a positive nitrogen balance occurred. Therapy was continued to the point of development of edema; slight hirsutism and masculinization of the voice were encountered. These subsided quickly after cessation of therapy and the skin lesions showed no recurrence four to five months after the end of therapy.

The Pulmonary Vascular Resistance. JOHN B. HICKAM (Introduced by Eugene A. Stead, Jr.), Durham, N. C.

Normally the pulmonary arterial pressure is low and little affected by changes in blood flow. In congestive failure the pressure is high and may be greatly increased by exercise without change in flow. It is difficult to interpret these observations in terms of the state of the pulmonary vessels because the pulmonary arterial pressure also depends on the blood flow and the pulmonary venous pressure. The latter can not usually be measured. The present report presents data obtained from 4 patients with atrial septal defect in whom it was possible to catheterize both pulmonary arterial and venous systems and to measure blood flow, pressure gradient, and pulmonary vascular resistance.

In 2 subjects without apparent pulmonary vascular disease, the resistance was extremely low (0.6 to 0.9 mm. Hg/l./min.). Blood flows of 15 and 20 l./min. were maintained by gradients of 13 and 12 mm. Hg. One subject with congestive failure and pulmonary arterial hypertension had a flow of 15 l./min. with a gradient of only 4 mm. Hg. This indicates that the high pulmonary arterial pressure resulted from transmission of a high pulmonary venous pressure back through the pulmonary vascular bed. The low gradient suggests passive dilatation of the bed. Exercise caused a large rise in pulmonary arterial pressure, two-thirds of which resulted from an increase in venous pressure and one third from an increase in vascular resistance. One subject had pulmonary vascular disease and a high resistance (80 times that of the preceding subject).

The observations provide quantitative data on the wide potential range of pulmonary resistance and suggest the means by which congestive failure produces pulmonary arterial hypertension.

Experimental Evidence on the Mechanism of Diabetic Ketosis. LAWRENCE E. HINKLE, JR., and GEORGE A. CONGER (by invitation) and STEWART WOLF, New York, N. Y.

In a study of 25 human subjects with diabetes mellitus, approximately 50 instances of clinical ketosis were observed to occur in a setting of emotional conflict and in the absence of other pertinent factors including infection. Moreover, day to day observation of these subjects both in and out of the hospital yielded a close correlation between life situation, emotion, and the metabolic state as

reflected by glycosuria, ketonuria, insulin requirement, and the symptoms of diabetes.

In an experimental study of nine of the subjects, quantitative measurements of blood ketone and glucose concentrations and concomitant determination of urine volume and glucose were made before, during, and after an interview in which intense emotional conflict was engendered. Ages of the patients varied from 14 to 60 years, and insulin requirements from none to 100 units per day. The chemical determinations were made on either peripheral venous blood or on blood withdrawn directly from a catheter introduced into the hepatic vein. In all cases a significant elevation of blood ketones, as well as a marked increase in the urine volume and rate of urinary glucose excretion, occurred during the traumatic interview. In the most severe diabetics the rapidity and degree of the increase in blood ketones and urine sugar was greatest, but nevertheless a marked degree of ketosis was produced in one of the mildest diabetics when the traumatic conflict situation was prolonged. The level of the blood glucose also fluctuated significantly, and was usually lower at the end than at the beginning of the experimental period.

Thus, significant emotional conflict has been shown to be associated with a rise in the blood ketone level and a simultaneous "washing out" of glucose through diuresis. The evidence indicates that such a mechanism is commonly involved in the decompensation of diabetes and the production of clinical ketosis.

The Usefulness of the Prompt Direct Reacting (1') Serum Bilirubin in the Study of Mild or Latent Jaundice. F. W. HOFFBAUER, Minneapolis, Minn.

During the last four years the fractional serum bilirubin determination according to the Ducci-Watson modification of the Malloy-Evelyn method, has been widely applied in the study of patients with hepatic, biliary tract, and hematologic disorders. The method is informative at any level of the serum bilirubin, but has demonstrated greater usefulness in mild or latent jaundice. Therefore, for the purpose of the present study, the cases included have had total serum bilirubins not exceeding 3 mg. per 100 cc.

The level of the prompt reacting (1') serum bilirubin has been very helpful in demonstrating hepatic abnormality or low grade biliary obstruction where the total serum bilirubin was increased but slightly or not at all. In a number of instances of minimal regurgitation jaundice, the increase in prompt reacting serum bilirubin was well correlated with bromsulfalein retention and positive cephalin cholesterol flocculation (parenchymal liver disease), and with increases of alkaline phosphatase and serum cholesterol (extrahepatic biliary obstruction). Conversely, in pure retention jaundice, increases in the indirect reacting (T-1') serum bilirubin, with slight or no increase in the 1' value, have been quite uniform.

Data correlating the essential clinical features and the results of other liver function studies will be analyzed and discussed.

Experimental Evidence on the Relative Effects of Life Stress and Inhaled Pollen in Hay Fever. THOMAS H.

HOLMES and THEODORE F. TREUTING (by invitation) and HAROLD G. WOLFF, New York, N. Y.

An experimental attempt was made to clarify the relative importance of stressful life situations and inhaled pollens in the pathogenesis of hay fever. In 30 subjects, 15 of whom had had hay fever, repeated quantitative studies of nasal function were made under uniform conditions in a room in which a known concentration of pollen was circulated.

Nasal hyperfunction characterized by varying degrees of hyperemia, swelling and hypersecretion of the membranes as well as eosinophilia in the nasal secretions and circulating blood occurred in all subjects, "sensitive" or "normal," exposed to mixed ragweed pollen when the circumstances were appropriate. When nasal function was average, neither "sensitive" nor "normal" subjects reacted to mixed ragweed pollen with sufficient evidence of nasal hyperfunction to produce symptoms. However, when there was preexisting nasal hyperfunction from whatever cause, both groups reacted to the pollen with marked nasal hyperfunction, weeping and sneezing. Thus during difficult life situations productive of conflict typical hay fever attacks followed pollen inhalation. Conversely, it was possible during pollen inhalation in the absence of frank hay fever to induce an attack by a discussion of significant personal problems and to induce subsidence of the attack by reassurance while the pollen is still being inhaled.

Unilateral procaine block of the stellate ganglion in 10 subjects yielded evidence that nasal hyperfunction engendered by conflict situations was mediated through parasympathetic fibers in the greater superficial petrosal nerve. Inhalation of pollen following this procedure precipitated the signs and symptoms of unilateral rhinitis which spared the non-hyperfunctioning membrane on the uninjected side. It was concluded that "sensitive" individuals differ from normals only in degree, that pollen or stressful life situations may induce nasal hyperfunction in either with associated local and circulating eosinophilia and symptoms of rhinitis. The various factors provocative of nasal hyperfunction with symptoms of hay fever thus exert an additive effect.

Quantitative Estimate of Vasomotor Tone in the Human Extremity with Comparative Studies of the Sympathetic Blocking and Adrenolytic Properties of Tetraethylammonium, Priscol, and Dihydroergocornine. SIPLEY W. HOOBLE, JOHN W. AVERA, SAMUEL G. MCCLELLAN and WILLIAM J. LITTLE (Introduced by Richard H. Lyons), Ann Arbor, Mich.

Presumably complete blockade of sympathetic vasomotor tone in the human subject without marked organic vascular disease was produced by means of spinal anesthesia, caudal anesthesia, lumbar paravertebral block, or within 24 hours after sympathectomy. Blood flow to the foot, as measured by the venous occlusion plethysmograph, was regularly increased to an average of 14 times control values. In contrast, tetraethylammonium (500 milligrams intravenously) increased blood flow to an average of 7 times resting levels and produced no vasodilatation after sympathectomy or after intra-arterial administration. In

usual clinical doses it therefore produced an approximately 50 per cent blockade of sympathetic vasoconstrictor tone and had no local vasodilator action.

Priscol (30-50 milligrams intravenously) induced a three fold increase in blood flow in the foot and a two fold increase in the innervated and denervated hand. Blood flow likewise was increased after intra-arterial injection. In this dosage the effects of the drug can in large measure be accounted for by a local vasodilator action, although slight sympatholytic activity can not be excluded.

Dihydroergocornine (0.25 - 1 milligrams intravenously) caused a slow and delayed increase in blood flow to 2.6 times resting levels in the foot and 2.1 and 2.4 times resting levels in innervated and denervated hand respectively. In this dosage the drug has little or no sympatholytic effects and may produce vasodilatation after conversion into a vasodilator agent or by altering existing vaso-regulatory mechanisms which are not mediated through sympathetic pathways.

Adrenolytic activity was measured by determining the effect of the drugs on the vasoconstrictor response to intra-arterial injection of epinephrin. When tetraethylammonium, DHO, or benzodioxane (16 milligrams) was administered intravenously, the response to intra-arterial epinephrin was not altered. On the other hand priscol intravenously had a moderate adrenolytic effect. When high local concentrations of the last three drugs were achieved by intra-arterial injection, significant adrenalin blocking action was demonstrated.

Iron Metabolism Studies in Normal Subjects and in Patients having Blood Dyscrasias. REX HUFF and THOMAS HENNESSEY (by invitation), and JOHN H. LAWRENCE, Berkeley, Calif.

Shemin and Rittenberg have provided a method for accurate determination of the life of the red cell. With the need in mind for a method of determining the rate of production of hemoglobin; iron turnover in plasma and red cells of normal subjects and patients having polycythemia, leukemia, and refractory anemia has been studied. Total subjects numbered 30.

Iron 59 (80-480 micrograms) was administered intravenously. The plasma iron level was not significantly altered; thus the clearance as measured by the rate of decrease of labeled iron in the plasma was effected at a constant plasma iron concentration. The coefficient of clearance (fraction of plasma volume cleared of iron per hour) was as low as 0.09 in a refractory anemia patient and as high as 3.84 in a polycythemia patient. The normal value was 0.35.

The appearance of iron 59 in the circulating red cells was usually of a double exponential nature. The time required for a final level to be reached varied from 5 to 40 days. The trend was toward a more rapid appearance in the polycythemics and some of the leukemics.

The fraction of the total dose present in the circulating red cells at equilibrium was computed on the basis of a circulating red cell mass determined by tagged cell methods or by estimation. The fraction utilized varied from 0.05 in a refractory anemia patient to 0.95 in a patient

who had had a severe hemorrhage. The average normal was 0.80.

The amount of iron incorporated into red cells per day varied from 220 mg. in a polycythemic to 2.9 mg. in a refractory anemia patient. The average normal value was approximately 20 mg.

The authors believe that here is a method for the determination of the rate of hemoglobin production in normal and pathologic states.

The Role of the Hypothalamus in the Pituitary-Adrenal Cortical Response to Stress. DAVID M. HUME (Introduced by Francis D. Moore), Boston, Mass.

The mechanism responsible for increased secretion of anterior pituitary adrenocorticotrophic hormone after non-specific trauma and stress, and the consequent rise in the titre of circulating adrenal cortical hormone has been studied in dogs and man. Trauma, insulin, chemical irritants, and epinephrine have been used as the stimulators of the pituitary-adrenal cortical system. The fall in circulating eosinophils has been used as an indicator of increased adrenal cortical activity, as this has been shown previously to be a measure of 11-17-oxysteroid output by the adrenal cortex, and to be dependent on the secretion of anterior pituitary adrenocorticotrophic hormone. The integrity of the anterior pituitary and adrenal cortex has been shown to be essential for the eosinopenic response to stress.

Anterior hypothalamic lesions in the supra-optic nucleus producing diabetes insipidus do not interfere with the normal eosinopenic response to stress; nor does divesting the pituitary of its vascular and nervous connections with the intact hypothalamus. However, specific localized electrolytic lesions placed in another area of the anterior hypothalamus abolish the usual eosinopenic response to stressing agents, even in the presence of an intact pituitary; and significant alterations in response follow lesions in the afferent tracts to this area. An extract of the hypothalamus has been prepared which on injection is capable of producing good eosinopenic responses in normal animals and also in animals whose hyperthalamic lesions render them incapable of responding to any of the stressing agents tested. These findings suggest the presence of a hormonal mechanism located in the anterior hypothalamus which, when activated in stress, will release a substance capable of causing an increased secretion of adrenocorticotrophic hormone by the anterior pituitary. In the absence of this mechanism no increased secretion of adrenal hormone can be detected in response to stress by the methods employed.

Electrolyte and Renal Function Studies in Lower Nephron Nephrosis. LLOYD T. ISERI, THOMAS M. BATCHELOR, A. J. BOYLE, and S. D. JACOBSON (by invitation) and GORDON B. MYERS, Detroit, Mich.

With the aid of the Beckman flame photometer and emission spectrograph, rapid determinations of plasma and urinary sodium and potassium were obtained and utilized as a guide to therapy in patients with lower nephron nephrosis. Simultaneous mannitol, para-aminohippurate,

sodium and potassium clearances were determined periodically. Impaired tubular reabsorption of sodium with relatively high urinary sodium concentration was demonstrated during the early stages and restoration to normal reabsorptive capacity during recovery. Markedly negative nitrogen balances were also observed during recovery.

In some of the cases, renal functional studies were correlated with the pathological findings in material obtained by renal biopsy or at autopsy. The relationship between pre-renal azotemia, lower nephron nephrosis, and bilateral cortical necrosis of the kidneys will be discussed.

Studies of the Principle in Liver Effective in Pernicious Anemia. VII. The Augmentative Effect of Accessory Factors on the Therapeutic Activity of Vitamin B12. BERNARD M. JACOBSON and (by invitation) RONALD C. BISHOP, Boston, Mass.

Evidence has accumulated that the therapeutic action of liver extract in pernicious anemia depends upon the presence of a *primary* factor, exerting by itself only slight or moderate hematopoietic effect, and upon the presence of five chemically distinct *accessory* factors which, in the absence of the primary factor, are therapeutically inert, but which augment the activity of the primary factor. The accessory factors include *l*-tyrosine, a peptide, xanthopterin, tryptophane, and guanosine.

The availability of crystalline vitamin B12 made possible the study of this material as a primary factor. Erythrocyte regeneration curves were constructed from data in the literature and from personal observations concerning sixteen patients who received vitamin B12 parenterally in a calculated average daily dose of 2.1 micrograms. When contrasted with similar curves representing the effect in a larger number of patients of commercial liver extract in a calculated average daily dose of 2.1 U.S.P. units, it becomes evident that the hematopoietic effect of pure vitamin B12 is inferior to that of a comparable dose of commercial liver extract. Erythrocyte regeneration following the administration of a comparable amount of non-crystalline primary factor together with accessory factors similarly was more rapid than that induced by vitamin B12. Thus, in a range of initial erythrocyte levels from 1.50 to 1.99 millions *per cu. mm.*, the average daily erythrocyte increment, in thousands *per cu. mm.*, were as follows: commercial liver extract, 78; primary factor together with accessory factors, 86; vitamin B12, 59. Similarly, in a range from 2.50 to 2.99 millions *per cu. mm.*, the average daily increment was 63, 61, and 31, respectively.

The augmentative effect of accessory factors on the action of vitamin B12 is illustrated by data of individual cases.

The Role of the Spleen in Radiation Injury. LEON O. JACOBSON, Chicago, Ill.

In an attempt to study the significance of the spleen in recovery from or compensation for radiation injury, four groups of mice were prepared. Group I consisted of untreated controls. Mice in groups II, III and IV were

anaesthetized with nembutal and the spleens brought out through an abdominal incision with the main pedicle intact. Groups III and IV mice were irradiated with 600 r whole body X radiation (250 kv.) except that during the irradiation the mobilized spleens of group IV were placed in a lead box with an opening for the pedicle only. After irradiation the spleens of groups II, III and IV mice were returned to the abdominal cavity.

The widespread destruction of hematopoietic tissue which developed in mice that received 600 r inclusive of the spleen (group III) produced a severe anemia, leucopenia and thrombocytopenia. Ectopic erythrocytopoiesis in the lead protected spleens of mice given 600 r (group IV) compensated with such rapidity for the marrow destruction that no anemia of significance developed. Ectopic granulocytopoiesis and megakaryocytopoiesis in the lead protected spleens compensated significantly but at a slower pace and less completely for the marrow destruction.

A marked and sustained decrease in the amount of lymphatic tissue occurred in the lead protected spleens (group IV) as ectopic hematopoiesis increased. This decrease in lymphatic tissue may be an indirect effect of irradiation.

The rapidity with which erythrocytopoiesis transferred from the X ray damaged marrow to the lead protected spleen in the absence of anemia suggests that the mechanism of stimulation of erythrocytopoiesis under the conditions of this experiment may involve some factor or factors other than or in addition to the accepted hemoglobin oxygen relationship.

Preliminary studies indicate that a significantly larger number of the irradiated animals with a lead protected spleen (group IV) survive than irradiated animals without spleen protection (group III).

Degradation of "Prothrombin-Converting Factor" in Sera of Individuals Receiving Dicumarol. RALPH F. JACOB and ROBERT BAYS (Introduced by Howard B. Slavin), Rochester, N. Y.

A study of the differential clotting action of *fresh serum* for fibrinogen and whole 0.01 M oxalated plasma, reveals that the serum coagulating factor is not thrombin, but a "prothrombin-converting factor." This factor requires ionized calcium before it can be activated from an inert precursor existing in oxalated plasma or circulating whole blood; while the conversion of prothrombin to thrombin by the serum factor takes place in the virtual absence of ionized calcium. Immediately after coagulation has been initiated by thromboplastin or platelets, a residuum of "prothrombin-converting factor" remains in the expressed serum. In individuals with a normal quantity of plasma prothrombin, the rate of disappearance (or degradation) of the serum "prothrombin-converting factor" is constant. In sera of individuals receiving dicumarol, the degradation rate of the serum "prothrombin-converting factor" usually varies directly with plasma prothrombin concentration. By applying the formula

$$\frac{\log C_1 - \log C_2}{T_1 - T_2}$$

wherein $ct_1 - ct_0$ is clotting time (seconds) of serum for plasma and $T_1 - T_0$ is elapsed time (minutes) for a given change to take place, one can express degradation rate (D.R.) as a unit.

The sera and plasmas of a series of patients receiving dicumarol were assayed daily for prothrombin (1 and 2 stage technique) and serum "prothrombin-converting factor" degradation rate. Good correlation between the two determinations existed, although the measurement of the degradation rate of serum "prothrombin-converting factor" was no substitute for conventional methods of prothrombin assay.

Immunologic Studies of an Iron Binding Protein in Human Serum. B. V. JAGER (Introduced by M. M. Wintrobe), Salt Lake City, Utah.

The immunologic properties of a crystalline β_1 globulin (Fraction IV-7-4) have been examined. This protein, isolated by Cohn and coworkers from plasma, has been found to have the property of binding with iron *in vitro*. A number of rabbits were immunized to this fraction. From a study of the antigen-antibody interactions of these antisera with the homologous antigen, Fraction IV-7-4 appeared to be immunologically homogeneous.

Standardized antisera were obtained by determining the amount of precipitate formed (antigen + antibody) after addition of varying known quantities of the IV-7-4 antigen. With this calibrated antiserum the amount of immunologically reactive IV-7-4 antigen in any unknown human serum can be determined by the quantitative precipitin technique.

As measured immunologically, the amount of IV-7-4 antigen in 14 normal human sera ranged from 0.21 to 0.31 grams per 100 ml. The mean value was 0.27 grams or 3.4 per cent of the total serum protein. Similarly, immunologic values were obtained for the sera of a number of pregnant women and a number of patients with acute or chronic infections. The values were increased in the pregnant women and reduced in the patients with infections. This was in accord with the alterations in the chemically determined total iron binding capacity of these sera.

The data are interpreted as indicating that the IV-7-4 protein present in human serum comprises all or nearly all of the iron binding protein.

Metabolic Changes in Heat Acclimatized Men during Abrupt Exposure to a Very Cold Climate. ROBERT E. JOHNSON and CHAUNCEY G. BLY (by invitation), ROBERT M. KARK and (by invitation) C. FRANK CONSOLAZIO, Chicago, Ill.

Thirty-two healthy young men, acclimatized for six months to Florida weather and unaccustomed to cold weather and its hardships, were taken by air from Florida (mean temperature $+57^\circ$ F.) to a very cold climate without layover, in January. On arrival in the subarctic, they were isolated in "survival areas." Studies were intensive for the first 24 hours (mean temperature -26° F.), and less intensive for 11 more days. Two questions were

asked. What metabolic changes are manifest in untrained, unacclimatized men during abrupt exposure to extreme cold, even with adequate clothing and environmental protection? For such changes can unifying explanations be found in the literature on cold and heat acclimatization, the catabolic phase after injury, or the "adaptation syndrome"?

Statistically significant responses during the first 24 hours included: transient hypothermia; diuresis, with negative water balance; hemoconcentration, with increases in serum protein, hemoglobin and hematocrit; eosinophilopenia and lymphopenia; neutrophil leukocytosis, with increase in the percentage of immature forms; hyperuricemia, hyperphosphatemia and hyperkalemia; hyponatremia and hypochloremia; transient retention of sodium and chloride, with transient hyperkalemia; and diminution of ascorbic acid in the blood and increase in the urine. Most of these changes reverted to normal in 48 hours. Statistically significant responses in the last six days of exposure included: increase in kidney and adrenocortical efficiency, a test dose of water being excreted rapidly at very low specific gravity; continued eosinophilopenia and lymphopenia; and continued hyperuricemia and hyperphosphatemia. Complete balance data yielded no convincing evidence of a catabolic phase.

The present changes have many similarities to those after injection of the adrenocorticotrophic hormone, and to the "general adaptation syndrome" of animals during stress.

Immunological Studies on Patients with Pneumococcal Pneumonia Treated with Penicillin. WILLIAM S. JORDAN (Introduced by John H. Dingle), Cleveland, Ohio.

Immunological studies of patients and animals with pneumococcal infections have shown that the naturally occurring production of antibodies is not affected by therapy with sulfonamide drugs. Meager data are available for patients treated with penicillin.

The immune response of 44 patients with pneumococcal pneumonia treated with penicillin was measured by agglutination and mouse protection tests. The results indicated that the antibody response of these patients was comparable to that occurring after spontaneous recovery or recovery following treatment with sulfonamides.

Two patients, one infected with type VIII and one with type I pneumococci, were treated within 2 and 3 hours of the acute onset of symptoms respectively. Neither patient developed a demonstrable pulmonary lesion or showed a rise in antibody titer. No other cases treated this early were observed. Of two cases treated within 7 hours of acute onset, both developed mouse protective antibodies and one developed agglutinins. Twenty-four cases received penicillin within 2 to 28 hours of the acute onset of symptoms. Nineteen of the 24 cases, or 79 per cent, showed demonstrable changes in antibody titers. This percentage response is comparable to that which occurred in the patients treated later than 28 hours after onset and parallels the response previously observed in patients not treated with penicillin.

Correlation of Changes in Renal Circulation with Metabolic Balances of Electrolytes and Nitrogen during Recovery from Congestive Cardiac Failure. A. KATTUS, A. GENECIN, J. H. SISSON, C. MONGE, B. C. SINCLAIR-SMITH, and E. V. NEWMAN (Introduced by Alan M. Chesney), Baltimore, Md.

Two edematous patients with congestive cardiac failure were followed during recovery on bed rest and digitalis with metabolic balances of sodium, chloride, potassium and nitrogen. Periodic determinations of renal plasma flow (RPF) and glomerular filtration rate (GFR) were made.

One patient was observed for four months, 24 days on metabolic balances. There was negative sodium and chloride balance for 14 days, the weight and venous pressure falling to normal. Potassium and nitrogen balances were markedly positive during negative sodium balance. More potassium was retained than could be theoretically associated with the nitrogen stored, possibly indicating repair of cellular damage incurred during congestive cardiac failure.

GFR remained within normal limits throughout the four months of observation. RPF rose and filtration fraction (FF) fell during recovery. Three months later after reaccumulation of edema, GFR remained normal, RPF had fallen and FF had risen.

Another patient showed marked sodium chloride loss in four days, the weight falling 4 kilograms, followed by nine days of sodium chloride retention without increase in weight. Equilibrium was then established. Exercise daily for the next 11 days caused seven days of sodium chloride retention, followed by equilibrium. There was potassium and nitrogen equilibrium throughout.

GFR was 50 per cent and RPF 33 per cent of normal with an average FF of 0.40 throughout the balance study. Later the patient's rhythm was reverted from auricular fibrillation to sinus rhythm whereupon GFR rose from 80 to 100 cc./min.

It is concluded that major changes in the body balances of sodium chloride and water may occur at both normal and reduced glomerular filtration rates. In each patient major shifts in salt balances, weight and venous pressure were not accompanied by significant change in glomerular filtration rate. The edematous state was associated with a high filtration fraction due to a low renal blood flow.

Factors Influencing Removal of Bacteria from the Blood Stream. GRACE P. KERBY, BERNARD C. HOLLAND and SAMUEL P. MARTIN (Introduced by W. C. Davison), Durham, N. C.

Factors influencing the removal of bacteria from the blood stream have been studied effectively by a method of combined venous catheterization and bacteremia induced by continuous bacterial infusion. The sites of removal of the bacteria are determined in the anesthetized dog from the bacterial counts of samples of blood removed simultaneously from femoral artery and from veins draining the organs studied.

The major removal of bacteria is accomplished in the splanchnic circulation, the liver and spleen showing equal

efficiency in the process. Removal during hind limb circulation may be of minor importance. No organisms are lost on circulation through the lungs.

Splanchnic removal rate is markedly and significantly decreased for a short period of time by thorotrast blockade.

The basic efficiency of the normally functioning removal mechanism varies widely with the bacterium used, being much greater in the dog for *Escherichia coli* and *Micrococcus aureus* than for *Klebsiella pneumoniae*. Arterial levels of 400 bacteria per ml. are reduced to 100 per ml. with each circulation through the splanchnic area. Sustained bacteremias clear within 15 to 30 minutes after bacterial infusion is discontinued.

Active immunization of the animal increases significantly the removal rate of *Klebsiella pneumoniae*. Administration of normal homologous plasma to the normal dog does not alter removal rate, but administration of equal amounts of homologous immune plasma results immediately in a striking increase in efficiency of splanchnic removal.

These alterations in efficiency of removal of bacteria from the blood stream unquestionably influence the ultimate course of the bacteremia. The extent of possible alteration and the exact mechanisms by which the removal is achieved and altered in the splanchnic area offer fruitful possibilities for further study. The present work points again emphatically to the importance of consideration of both parasite and host factors in any such continued investigation.

Studies in Protein Metabolism with the Aid of S³⁵-Labeled-Methionine. LAURANCE W. KINSELL, SHELTON MARGEN, HAROLD TARVER, JULIE MCB. FRANTZ and ERIN K. FLANAGAN; with the technical assistance of VERNON T. THOMPSON and ROBERT V. DEAL (Introduced by T. L. Althausen), Oakland, Berkeley and San Francisco, Calif.

S³⁵-labeled-methionine has been administered to normal males and to patients with the following entities: (1) Chronic Liver Damage, (2) Idiopathic Hypoproteinemia, (3) Cushing's Syndrome. Incorporation of the labeled material in plasma protein, as well as its excretion in urine and stool, have been quantitated (in most instances as one part of protein balance studies).

From the results obtained, one may tentatively conclude that both the idiopathic hypoproteinemia syndrome and Cushing's syndrome are characterized by a more than normal rate of initial plasma protein formation (as evidenced by incorporation of S³⁵) followed (certainly in the former and probably in the latter) by excessive catabolism, at a rate such that net protein deficiency results.

The three patients with chronic liver damage so far studied show a diminished rate of incorporation of the labeled material into plasma protein, and a subsequent rate of disappearance not unlike that observed in the normal.

Urinary sulfate excretion data are in excellent accord with the preceding observations.

Chloromycetin in the Treatment of the Acute Manifestations of Brucellosis. VERNON KNIGHT and FRANCISCO

RUIZ-SANCHEZ (Introduced by Paul Reznikoff), New York, N. Y.

Twelve adult patients with acute manifestations of brucellosis have been treated with chloromycetin. All but one of these individuals were treated in Guadalajara, Mexico, and the majority of the infections were caused by *Br. melitensis*. All of the patients were febrile and acutely ill when antimicrobial therapy was started. The pretreatment illness was less than 90 days in 8 of the patients and ranged from 7 to 12 months in the remainder.

The diagnosis of brucellosis was established by demonstration of bacteremia in six patients and by a significant elevation in antibody titer in the others.

Appropriate clinical, bacteriologic and serologic observations were made during the acute illness and during the ensuing three months.

In general, chloromycetin was administered for six or ten days, in daily dose of 50 to 100 mg. per kg. while febrile, and 25 mg. per kg. thereafter.

Clinical improvement after therapy was dramatic. In every case temperature and symptoms rapidly diminished in the first three days of therapy. The average duration of fever was only 2.0 days, except for one individual who made an unsatisfactory response after six days of treatment with low doses. When retreated with higher doses, his course was comparable to that of the other patients.

The chloromycetin results can be compared with results observed following the aureomycin treatment of brucellosis by Spink and his associates and by the present writers. The immediate response of the patients treated with chloromycetin was indistinguishable from the response observed after aureomycin therapy.

Relapse was not uncommon following short periods of therapy with either drug. In the chloromycetin-treated series, relapse occurred within six weeks of the cessation of therapy in five of the twelve cases. The effect of a considerable prolongation of therapy upon the incidence of relapse is under investigation at present.

The Effect of Radiation on Total Coproporphyrin Excretion. HENRY J. KOCH, JR., TIMOTHY R. TALBOT, JR., and MITCHEL BERNSTEIN (Introduced by Konrad Dobriner), New York, N. Y.

The rate of total coproporphyrin excretion in the urine and feces has been observed in several patients receiving either the 8-day half-life radioactive isotope of iodine or x-radiation. In conjunction with this study complete hemograms including serial red-cell counts, hemoglobin, white-cell counts, platelet counts, bleeding and clotting time, bone-marrow biopsy, and liver function tests have been followed.

Three-day aliquots of urine and feces have been extracted for total coproporphyrin content according to the methods established by Dobriner and Watson. The etioisomers I and III of coproporphyrin are to be differentiated in a separate study.

It has been noted that there is an increase in total coproporphyrin excretion following radiation. This effect has been observed in patients receiving radioactive iodine

and x-radiation given in therapeutic doses, the largest single dose being 112 millicuries of I^{131} . The increased excretion has been reflected in both the urine and feces and has occurred ten to fourteen days following the onset of irradiation.

Concomitant with this increase in coproporphyrin excretion, a decrease in total lymphocyte count and a transient decrease in platelet count have been observed.

Granulomatous Polyarteritis: Report of a Case with Intra- and Extravascular Granulomas Combining Certain Morphological Features of Polyarteritis Nodosa and Acute Rheumatic Fever. J. P. KULKA, D. G. FREIMAN and W. S. CLARK (Introduced by William W. Beckman), New York, N. Y.

The relation between vascular and extravascular lesions is one of the major problems in the morphology of the rheumatic diseases. Of particular interest is the relation between the characteristic necrotizing angiitis of polyarteritis nodosa and the Aschoff bodies and other extravascular granulomatous lesions of rheumatic fever. While both human autopsy studies and animal experiments seem to point to an intimate connection between these two disease manifestations, no direct evidence has yet been produced to indicate whether they are the result of the same or of different types of tissue injury.

Observations are therefore reported on a case of bronchial asthma and polyarteritis nodosa which at autopsy showed all transitional stages from classical segmental panarteritic lesions to miliary intramural granulomas on the one hand, and from extravascular miliary granulomas identical with those in the vessels to lesions indistinguishable from typical Aschoff bodies on the other. These findings strongly suggest that the segmental angiitis and the focal granulomas are both produced by the same pathogenic mechanisms and provide additional evidence that polyarteritis nodosa and rheumatic fever may be expressions of the same fundamental disease process.

Effect of Rigid Na Restriction in Patients with Cirrhosis of the Liver and Ascites. HENRY G. KUNKEL, WILLIAM J. EISENMENGER and EDWARD H. AHRENS, JR. (Introduced by Thomas M. Rivers), New York, N. Y.

The intake of NaCl was limited to less than 1 gm. per day in 13 patients with steadily accumulating ascites. Fluid formation in the abdomen was immediately stopped in 12 of the 13 patients. The patients readily tolerated such a low salt diet for more than 3 months with the exception of one patient who showed an elevated NPN initially. As much as 150 gm. of protein per day could be consumed on this diet. The 12 patients who ceased to form fluid were markedly improved by the treatment, chiefly because the loss of body protein through paracentesis ended. This resulted in a rise in the serum albumin level. However, ascites reformed in all but 3 of the patients when the Na intake was raised to normal levels at the end of 3 months and longer periods of therapy were necessary for complete improvement. The critical level of NaCl intake where ascites formation began was ap-

proximately 1.3 gms. NaCl per day. Above this level the amount of ascites which formed was directly proportional to NaCl intake.

Detailed studies of nitrogen, Na and K balances were carried out in 3 of the patients before and after Na restriction. The patients were found to be in negative nitrogen balance prior to therapy chiefly because of the loss of ascitic fluid nitrogen. When this was stopped, they reverted to positive nitrogen balance. All the patients showed extremely low excretion of Na in urine prior to therapy and this was further reduced after the special diet was begun. Fecal Na levels were approximately 3 times the urinary values.

The conclusion drawn from these investigations is that rigid Na restriction is a valuable supplement to nutritional therapy for stopping the malignant downhill course of patients with rapidly accumulating ascites.

Studies of Water Storage in Liver Disease. II. The antidiuretic activity of the urine during acute infectious hepatitis. DANIEL H. LABBY (Introduced by Edwin E. Osgood), Portland, Ore.

In a previous study of 14 cases it was demonstrated that the acute phase of infectious hepatitis was marked by expansions of the thiocyanate fluid space, the plasma and blood volumes, depression of the plasma and urinary chlorides and increased water tolerance. Improvement in liver function with convalescence resulted in shrinkage of these fluid compartments and diuresis. Slight changes in total circulating proteins were detected. Eight additional cases of early hepatitis were selected to correlate the antidiuretic activity of the urine with the above fluid observations. 200 gm. male rats were given water (5 per cent of body weight) by stomach tube and injected intraperitoneally with 1 cc. of the patients dialized and concentrated urine per 100 grams of rat. The time required for the excretion of 50 per cent of the ingested water was taken as a standard assay end point; controls were run simultaneously by injecting water of the same pH intraperitoneally. The data obtained in each case indicate that in the early phase of acute infectious hepatitis at the time of maximum water storage and increased water tolerance the 50 per cent excretion time ranged from 155 to 360 minutes in comparison to control times of 80 to 110 minutes. With convalescence, contraction of the thiocyanate space, and diuresis, the antidiuretic titer of the urine decreased to control values. It is therefore concluded that the movements of body water occurring during acute infectious hepatitis are accompanied by alterations in the concentration of the antidiuretic principle in the urine; the potency of this principle being highest during periods of water storage in the acute stage and lowest during convalescence when excessive water storage is no longer demonstrable. Opportunity was also presented for conducting these studies during menstruation in the course of hepatitis and in a case of nonicteric hepatitis.

Evidence That Renal Sodium Excretion Is Controlled by Adrenal Cortical Activity and That Ingested Sodium May Displace Intracellular Potassium in Normal Sub-

jects. ALEXANDER LEAF (by invitation) and L. H. NEWBURGH, Ann Arbor, Mich.

The effects of great variation in sodium intake were studied in normal subjects maintained on fixed diets of adequate calories and protein with very low sodium and chloride content. At definite periods additional sodium was administered either as the chloride or citrate.

Salt restriction resulted in reduction in urinary sodium and chloride to minute amounts accompanied by increase in urinary nitrogen, urea, uric acid, potassium and phosphorus. Administration of sodium chloride or citrate caused high urine sodium with decrease in urine uric acid, potassium phosphorus, blood and urine urea, and positive nitrogen balance. The drop in blood urea was not accounted for by alteration in glomerular filtration rate but was explained by decrease in the rate of protein catabolism. A strongly positive potassium balance occurred simultaneously with the high sodium excretion that could not be accounted for by decreased protein catabolism.

All these changes are explained by alterations in adrenal cortical activity. The need to conserve body sodium was met by increased activity of the desoxycorticosterone-like hormone. An associated increase in protein-catabolic hormone activity was elicited also. Sodium administration abolished the need to conserve sodium and thus depressed adrenal cortical activity. This not only allowed a large urinary sodium excretion but also gave evidence of a marked decrease in activity of protein-catabolic hormone.

In all subjects sodium citrate administration caused initially a marked positive sodium balance. The degree of alkalosis was reduced by entry of large amounts of sodium into the cells with displacement of large amounts of potassium as evidenced by the strongly negative potassium balance.

Diagnosis of Carcinoma of the Liver, Biliary Tract, and Pancreas from Cytology of Duodenal Aspiration. H. M. LEMON and W. W. BYRNES (Introduced by James M. Faulkner), Boston, Mass.

The infrequency of successful surgical therapy for primary carcinoma of the liver, extra-hepatic bile ducts, and pancreas reflects in many cases clinical inability to establish an early positive diagnosis of cancer. In this study careful cytological examination has been made of aspirated duodenal contents in an effort to supplement currently available diagnostic methods. Although no previous studies have been reported concerning neoplastic cells in duodenal secretion, we have found that criteria accepted as indicating cancerous cell exfoliation from other epithelial surfaces can be readily applied to this fluid, since large numbers of tumor cells as well as normal epithelial cells have been observed in the majority of our specimens from patients with early or late cancer.

All cases have been followed for at least six months after examination or until death. Three-quarters of the cancer cases have been verified pathologically, and a close similarity in morphology has been found between the exfoliated cells and those in the pathological sections from the tumor. No false positive errors have been made, although control observations have been carried out in an equally

large number of cases of cirrhosis and inflammatory condition of the biliary tract and pancreas. Among the diagnosed cancers were hepatoma, primary cancers of the gall bladder, common bile duct, Ampulla of Vater, and the head and tail of the pancreas. Localized carcinomas as small as 2 cm. in diameter have been detected, as well as certain metastatic neoplasms. A brief description will be given of certain cases in which earlier diagnosis and institution of proper therapy have resulted from the application of this method to clinical problems with and without obstructive jaundice.

Acute Asphyxia in Dogs: Effects on Blood Coagulation and Citric Acid Metabolism. JESSICA H. LEWIS (Introduced by E. C. Hamblen), Chapel Hill, N. C.

These experiments were designed to study the effects of acute asphyxia on blood coagulation in dogs. The trachea was clamped in 13 anaesthetized dogs. Following the cessation of labored respiratory movements at about two and one-half minutes, the blood pressure fell, reaching zero at approximately six minutes. Two cc. arterial whole blood specimens were collected every fifteen seconds for the first two minutes and every five seconds thereafter. Markedly prolonged clotting times or incoagulable bloods were obtained during a 30-45 second interval occurring about 30 seconds after respirations had ceased. Both before and after this period blood coagulation times were normal.

Blood coagulation studies on the "incoagulable blood" showed normal (1) prothrombin complex, (2) fibrinogen, (3) platelets, (4) fibrinolytic enzyme system, and (5) antithrombin. No heparin or defect correctable by added thromboplastin could be detected.

The coagulation defect was readily reversed by the addition of calcium chloride. In spite of this, the total calcium concentration was normal. Therefore, we reasoned that calcium must be bound in some soluble, but non-ionized form.

Anaerobic carbohydrate metabolism may produce citric acid. Studies on four dogs have shown a rise of citric acid concentration from a normal value of approximately 75 micrograms/cc. to 1,000 micrograms/cc. in the "incoagulable blood" followed by a fall to normal values at death. Aconitic acid did not change appreciably. Parallel studies with added sodium citrate showed that 900 micrograms/cc. (as citric acid) were sufficient to prevent coagulation.

Acute asphyxia in the dog produces a marked rise in blood citric acid content, sufficient to bind ionized calcium, and so far as we are aware, this is the first reported incidence of calcium ion deficiency resulting in a blood coagulation defect. An adequate explanation for the extremely temporary but marked elevation in blood citric acid is being sought.

Studies in Urinary Corticosteroids by a Method Permitting Analysis of Desoxycorticosterone. CHARLES W. LLOYD and JULIA LOBOTSKY (Introduced by Eugene L. Lozner), Syracuse, N. Y.

The method of Daughaday, Jaffe and Williams for estimation of freely water soluble urinary corticosteroids has been modified to permit analysis of the poorly water soluble corticosteroids, such as desoxycorticosterone.

Excretion of corticosteroids by normal individuals and by six patients with Addison's disease has been studied by this method. Low corticosteroid values were found in all patients without treatment. Following beginning of desoxycorticosterone acetate injections, there was a latent period of several days before the excretory rate began to rise. In one patient with a fixed daily dose of 5 milligrams of desoxycorticosterone there was a gradual increase in corticosteroid excretion over a period of a month. Following complete withdrawal of desoxycorticosterone, excretion continued at a high level for approximately 96 hours before a significant decrease was observed. In one patient, clinical evidence of adrenal insufficiency appeared within one day of this decrease. The excretion rate of corticosteroid was in the middle of the normal range when the desoxycorticosterone dose was at the optimal level as determined by clinical response. Excessive dosage was associated with higher corticosteroid values, which, however, did not exceed the normal range. Following implantation of pellets, with good clinical control, excretion of corticosteroid was lower than when desoxycorticosterone in oil was injected in amounts sufficient to produce a comparable clinical effect.

One patient with Addison's disease when untreated had a corticosteroid excretion only slightly below the normal range. Administration of desoxycorticosterone produced relatively small increases of urinary corticosteroids. Withdrawal of desoxycorticosterone was followed by the very slow appearance of symptoms of insufficiency. It is believed that this patient has viable adrenal tissue which is capable of secretion, although at an inadequate rate, and it is suggested that when desoxycorticosterone was administered, this secretion was depressed.

Studies in Hemoglobin Formation with the Aid of the Isotope Technique. IRVING M. LONDON, DAVID SHEMIN and D. RITTENBERG (Introduced by Randolph West), New York, N. Y.

When the whole blood of patients with sickle cell anemia is incubated *in vitro* with glycine labeled with N^{15} , heme containing N^{15} is formed. This finding demonstrates *in vitro* synthesis of heme. No significant synthesis is found on incubation of normal human blood. To help determine whether the immature reticulocyte or the mature pathologic cell of sickle cell anemia is responsible for the synthesis, studies have been carried out in a variety of hematologic disorders characterized by elevated reticulocyte counts (pernicious anemia, congenital hemolytic jaundice, acquired hemolytic anemia) and in experimental animals in whom reticulocytosis was induced by bleeding or phenylhydrazine hemolysis. These studies indicate that some, though perhaps not all, reticulocytes are capable of synthesizing heme *in vitro*. The differences in capacity for heme synthesis manifested by reticulocytes of various sources will be discussed.

The incubation of N^{15} labeled glycine and other isotopic

compounds with blood containing numerous reticulocytes affords a method for the study of heme formation. Avian nucleated red blood cells may be used similarly. Studies concerned with the optimal conditions for *in vitro* heme synthesis, with the use of the *in vitro* systems for the determination of the biologic precursors of protoporphyrin, and with some of the mechanisms involved in heme formation will be discussed.

Experimental Airborne Influenza Virus A Infection in Immunized Animals. CLAYTON G. LOOSLI and (by invitation) ROBERT S. ROCKWALD and MERLE H. RITTER, Chicago, Ill.

Young adult Swiss mice (10-15 grams) were immunized by 4 weekly 0.1 ml. subcutaneous injections of formalized mouse lung suspension (10-1 dilution) of PR-8 strain of Influenza A virus. Neutralization and CRC agglutinin-inhibition tests were employed for antibody determinations on pooled sera. Immunized and nonimmunized mice of similar age were exposed in a chamber at the same time to a lethal atmosphere of nebulized mouse lung PR-8 virus suspension. Five mice from each group (2 for histology and 3 for virus and antibody titration) were bled and killed at close intervals until death or 16 days after inoculation. Additional animals served to determine survival rates.

In the non-immunized animals, the influenza infection was rapidly fatal with death occurring on or before the fifth day. The lungs at death showed extensive consolidation with marked destruction of the bronchial epithelium and extensive edematous and cellular exudate in the alveoli. The immunized animals became moderately ill but survived. Grossly, only focal areas of consolidation occurred, which underwent resolution after 8 days. Microscopically, as in the control lungs, there was extensive destruction of the bronchial epithelial cells but the alveolar exudate was patchy and generally confined to regions near the terminal bronchioles. Also, as in the non-immunized animals, the virus grew rapidly in the lungs of the immunized animals, reaching a titer of over 10^6 lethal intranasal doses at 36 and 48 hours. At this time, the serum antibody level of 1:512 before infection had dropped to the non-immune level of < 1:16. At 6 days, only traces of virus were present and the antibody titer had risen several fold above the original immune level. The mechanism of protection against influenza virus in the immunized animals and in man in relation to the above observations will be discussed.

Spirocheticidal Antibodies in Syphilis. HAROLD J. MAGNUSON and (by invitation) FRED A. THOMPSON, Chapel Hill, N. C.

The *in vitro* technic described by Nelson, employing the Nichols strain of *T. pallidum*, permits demonstration of a spirocheticidal substance found in most syphilitic sera from humans and rabbits but not present in normal sera. Electrophoretic fractionation of such sera shows the activity to be associated with the gamma globulin. The substance is found in Fraction II-III of Cohn, is relatively

heat stable, and requires complement for its demonstration. This evidence suggests that the substance is an antibody.

The antibody is not related to reagin since its presence bears no relation to the titer of serologic tests for syphilis. Sera from which reagin has been absorbed retain their activity, and the purified reagin recovered from the precipitate is inactive. Assays are reported on sera from 200 patients with syphilis in various stages as well as from rabbits with known varying degrees of immunity. The possible relationship of this antibody to acquired immunity is discussed.

The Production of Renal Glomerular Lesions in the Diabetic Rat. GEORGE V. MANN and JAMES W. GODDARD (Introduced by Howard F. Root), Boston, Mass.

Weanling male rats have been made diabetic by the intravenous injection of alloxan monohydrate. A total of 50 animals have been maintained on one of 4 diets up to 550 days. The diets were dog chow and semipurified diets containing 12, 18 and 40 per cent protein. No insulin was given. At intervals animals were sacrificed and tissues examined.

It has been found that diabetic rats develop well marked progressive lesions in the renal glomeruli as early as 50-90 days. The lesions primarily affect the reticulin walls of the glomerular capillary tufts, parietal layer of Bowman's capsule and tubular basement membranes.

Reticulin proliferation produces intercapillary adhesions and luminal obliteration. The latter process may occur either by collapse reticulosis or by luminal ingrowth of reticulin fibers. In some instances hyaline-like balls are entrapped in the inter-capillary and axial spaces. Reticulin overgrowth in a meshwork pattern leads to ball formation, reminiscent of the fibrous balls of human nephrosclerosis. The parietal layer of Bowman's capsule shows irregular thickening and collagenization. Tubular basement membranes show thickening with hyaline changes.

Especially in old diabetic animals an increased cellularity of the glomerular tufts appears due to a remarkable hyperplasia and hypertrophy of what appear as perithelial cells.

These lesions have not been found in nondiabetic control rates or in nondiabetic alloxan injected controls. No lesions have been found in the aorta and its branches. Renal arteriolar lesions have been minimal. Neither the severity of diabetes nor the level of protein in the diet has been shown to influence these manifestations.

Mechanism of the Depressor Effect of Accelerated Sodium Depletion in Hypertension. R. S. MCGROW, J. J. BOORMAN, J. SIFOTA and H. POLLACK, New York, N. Y.

We have shown that the depressor response developing in hypertensive patients on low sodium diets may be accelerated materially by increasing the rate of sodium depletion through the use of mercurial diuretics.

The following studies were undertaken in order to elucidate the more fundamental relationships involved:

A: Adrenocortical function was investigated from determinations of the 17 keto and 11 oxy corticosteroids.

from glucose tolerance tests, from the blood uric acid, and from the eosinophile and lymphocyte response.

B: The effects of sodium depletion upon glomerular and tubular function and upon renal blood flow were determined by measuring the para-amino-hippurate and the mannitol or inulin clearances.

C: Using the T-1824 method, the possible role of decreased circulating blood volume in the depressor response to sodium depletion was studied.

D: The time relationships between sodium depletion and the depressor response were clarified by measuring the daily 24-hour sodium excretion by the flame photometer method.

E: Alterations in vascular reactivity were evaluated microplethysmographically, by contrasting the vasodilator responses to tetraethylammonium and dihydroergocornine before and after accelerated sodium depletion.

The evidence suggests that the decline in blood pressure which follows accelerated sodium depletion is associated with a temporary alteration of corticoid function. The conclusions drawn from the above investigations will be illustrated and discussed more completely.

Clinical Studies on the Pure Veratrum Alkaloids Protoveratrine and Veratridine. EDWARD MEILMAN and OTTO KRAYER (Introduced by S. L. Gargill), Boston, Mass.

The vasodepressor and cardiodecelerator action of *protoveratrine* hydrochloride has been studied for the first time in human renal and essential hypertension. It was administered intravenously, eighty times in fifteen patients, in single doses of between 0.08 mg. and 0.18 mg.

The characteristic response is a fall of systolic and diastolic blood pressure reaching minimum level within ten minutes. Intensity of effect is roughly proportional to the dose, varying between a significant decrease and a blood pressure fall to normal levels. Duration of action, likewise, is proportional to the dose, return to hypertensive levels occurring between fifteen minutes and three hours. Repeated administration of the same effective dose, in the same patient, leads to similar responses after the previous effect has worn off.

The vasodepressor action is accompanied by bradycardia, usually with regular sinus rhythm. Occasionally, the largest doses shifted the pacemaker, the ventricle beating faster than the auricle.

Atropine reduces but does not abolish the vasodepressor effect, while it increases the heart rate above normal and restores regular sinus rhythm. Simultaneous administration of *protoveratrine* and atropine causes a vasodepressor effect with a concomitant increase in heart rate.

Apart from the disturbance of rhythm, a slight and transient feeling of nausea occurred a few times.

Veratridine has circulatory effects qualitatively similar to those of *protoveratrine* but is less potent. With *veratridine* significant vasodepressor action cannot be obtained without side effects like nausea, vomiting, sweating, and feeling of apprehension. Occasionally, these occurred with doses not decreasing blood pressure.

Their unique mechanism of action makes the *Veratrum* alkaloids important substances for careful clinical studies in hypertension. For satisfactory clinical use the search must be directed towards pure alkaloids pharmacologically similar to *protoveratrine*, with a margin of safety and an intensity and duration of vasodepressor action equal or possibly superior to that of *protoveratrine*.

The Determination of Maximum Composite Digital Vascular Caliber Physiologically in Normal and Hypertensive Subjects. MILTON MENDLOWITZ, New York, N. Y.

In animal perfusion experiments with Ringer's solution the pressure-flow relationship has been demonstrated to be rectilinear. For blood this relationship has been found to be logarithmic. That portion of the curve above flow readings of one-third the "normal," however, corresponds closely to a rectilinear tangent with an intercept on the pressure coordinate above zero.

The pressure-flow relationship in the human digital circulation after release of sympathetic tone was studied at various pressure levels achieved by graded compression of the brachial artery. This relationship corresponded well with a rectilinear tangent both in normotensive and hypertensive subjects. The intercepts were similar to those observed in animal experiments.

The "apparent viscosity" of normal blood at a "normal" perfusion pressure has been shown in animals to be 2.2 times that of Ringer's solution. There is reason to believe that this holds for the human digital circulation. If the intercepts are known, it is possible to draw a pressure-flow tangent from a single pressure-flow reading. At the point of "normal" pressure a theoretical Ringer's solution perfusion point can be erected and the Ringer's solution pressure-flow line drawn to the intercept zero. Poiseuille's law is now applicable, the only unknown quantities being length and caliber of the perfused vessels. Since the flow is measured per sq. cm. of skin, the average length of the perfused vessels can be estimated at 1.1 cm. The composite digital vascular caliber of a single vessel, the resistance of which is equal to that of all the vessels per sq. cm. of skin perfused can be computed. It was found to vary from 67 to 75 micra normally and to be below normal in hypertension.

Clinical Experience with the Use of an Artificial Kidney. J. P. MERRILL (by invitation), G. W. THORN, and (by invitation) E. J. CALLAHAN and S. SMITH, Boston, Mass.

A modification of the Kolff type of artificial kidney has been used in 28 cases in 18 patients with uremia. Difficulties encountered by previous observers with hemolysis, clotting and pyrogen reactions have been virtually eliminated. Adjustment of the tonicity of the bath fluid to the patient's serum by means of freezing point determinations has so greatly increased the accuracy with which hydration may be controlled that it is feasible to employ the procedure in uremic patients with congestive failure and even pulmonary edema. The adjustment and balance of blood flow rates through the machine have further de-

creased its risk in application to patients with cardiovascular disease.

The effectiveness of this machine in removing diffusible substances from the blood by in vivo continuous dialysis is indicated by the fact that with blood flow rates between 200 and 300 cc. per minute and blood urea nitrogen level of 80 to 90 mg. per 100 cc., it is possible to remove 20 grams of urea per hour with comparable quantities of other retained metabolites. The machine may also be employed to remove specific ions by dialysis and three instances of acute spontaneous potassium intoxication have been successfully treated by the removal of excess potassium from the blood stream. The possibility of removing specifically other diffusible substances, including sodium, uric acid, water and amino acids is under investigation. It is concluded that the present technique of application of an artificial kidney is an effective and clinically safe procedure for the removal of diffusible substances by continuous dialysis of the blood. The value of its use as a means of therapy in acute renal failure and in a variety of forms of intoxication is well established. Its use as an investigative tool in the study of chronic cardiovascular and renal diseases is apparently warranted.

Treatment of Pernicious Anemia with an Animal Protein Factor of Bacterial Origin. LEO M. MEYER, NORTON D. RITZ, MANUEL ROWEN, GEORGE BOCK and JULIUS RUTZKY (Introduced by Arthur C. De Graff), New York, N. Y.

An animal protein factor concentrate was prepared from the aerobic fermentation of a non-motile gram negative bacillus isolated from chicken feces. One cc. of this product (A.P.F. 60) had a biologic activity equal to "10 unit liver" when administered to chicks receiving a diet containing 70 per cent soy bean meal. In the lactobacillus assay the material had a somewhat lower potency (65 per cent of "10 unit liver"). A more concentrated preparation (Normocytin) had a potency comparable to 10 micrograms of vitamin B 12 when administered to chicks maintained on a diet containing 70 per cent soy bean meal. Administration of A.P.F. 60 to 5 patients with pernicious anemia in relapse induced a satisfactory reticulocyte response in 4 instances, with an increase of Hb and R.B.C. in all cases. Eight other persons with pernicious anemia in relapse treated with Normocytin showed a satisfactory reticulocytosis. An increase of Hb and R.B.C. to normal levels occurred in 6 cases. One patient from the original group treated with A.P.F. 60 was continued on Normocytin and also reached normal hematologic values. In all instances there was improvement in appetite and general well-being of the patients. Megaloblastic bone marrows were converted to normal states. Where neurological changes were present improvement was noted. There was no instance of progression or development of nervous system signs or symptoms. In two patients the Hb and R.B.C. remained below normal levels and are at present under treatment with vitamin B 12.

Effect of Urethane on the Susceptibility of Mice to Pneumonia Virus of Mice (PVM). GEORGE S. MIZICK,

CHARLES I. LEFTWICH, JR. and WILLIAM B. LEFTWICH (Introduced by Paul W. Clough), Baltimore, Md.

The effect of urethane on the susceptibility of the young mouse to infection by pneumonia virus of mice (PVM) was tested. This compound seemed of interest because of its reported suppressive action on cell growth and its capacity to induce pulmonary tumors in older mice of certain strains.

Suitable matched groups of young mice were lightly anesthetized with ether, and inoculated intranasally with serial ten-fold dilutions of PVM. All groups were fed Purina Dog Chow and each was given to drink either water or water containing various concentrations of urethane. It was observed repeatedly that drinking 0.1 per cent or more urethane during the post-inoculation period rendered mice 5 to 10 times more susceptible to this virus infection than controls. A single intraperitoneal injection of urethane before virus inoculation produced similar effects.

Some possible mechanisms for urethane's action in increasing the mouse's susceptibility to PVM were studied. Mice given urethane orally gained less weight and drank less fluid than controls. However, limiting food or fluid in other mice did not reproduce the effect of urethane. Moreover, one injection of 5 mg. of urethane before inoculation increased susceptibility ten-fold without affecting the weight curve. Other cell growth depressants like colchicine and podophyllotoxin did not have effects like that of urethane on susceptibility to the virus infection.

Insulin Tolerance Test in Patients with Essential Hypertension. I. ARTHUR MIRSKY, (by invitation) STANLEY M. KAPLAN and R. H. BROH-KAHN, Cincinnati, Ohio.

The blood sugar response to the intravenous injection of a standard dose of insulin is dependent upon factors which affect the hypoglycemic action of insulin and factors which affect the restitution of the blood sugar to the pre-injection level. Some of the hormones of the anterior pituitary gland and the adrenal cortex are among the factors which determine the effectiveness of the hypoglycemic action of insulin whereas the activity of the sympathico-adrenal system largely determines the rate of restitution of the blood sugar concentration to the preinjection level. Since both sets of factors have been implicated in the genesis and maintenance of the hypertension of patients with essential hypertension, we deemed it pertinent to study the insulin tolerance of such patients.

Blood samples were drawn for sugar determination before, and at 15, 30, 45, 60, 90 and 120 minutes after the intravenous injection of 0.1 unit insulin per kilogram body weight to fasting normotensive and hypertensive subjects. The carbohydrate stores of all subjects were fortified by ingestion of two grams of carbohydrate per kilogram body weight after the usual meal the night before the test.

The hypoglycemic response of both groups of subjects was not statistically different. However, statistical analysis of the data revealed that the rate of restitution of

the blood sugar concentration to the preinjection level was markedly delayed in the hypertensive group.

These studies indicate that there is no significant increase in contra-insulin factors in patients with essential hypertension. Further, if the rate of restitution of the blood sugar concentration is dependent upon the degree of activity of the sympathico-adrenal system, then the response of this system to hypoglycemia must be reduced in the hypertensive group.

The Destructive Effects of the Virus of Russian Far East Encephalitis on the Transplantable Mouse Sarcoma 180.

ALICE E. MOORE (Introduced by C. P. Rhoads), New York, N. Y.

A study was made of the ability of certain viruses to grow in transplantable mouse tumors and to determine what effect their growth would have on the viability of the tumor. The virus of Russian Far East Encephalitis was found to have a marked affinity for the transplantable mouse Sarcoma 180 and in the process of viral infection the viability of the tumor was completely destroyed. When subinfective amounts of virus were inoculated there was no effect on tumor growth and no virus could be recovered from the tumor or brain.

Following intraperitoneal inoculation of 0.05 cc. of 10^{10} brain virus suspension, tumors were removed for bioassay and the amount of virus present in the blood, brain and tumor was determined. For the first five days more virus was present in the tumor than in either blood or brain. From the 5th day until the death of the animals approximately equally large amounts of virus were present in the tumor and brain while it disappeared from the blood. Portions of the tumors were implanted into virus-immune and non-immune control mice at different days after intraperitoneal inoculation. In all cases in which implantations were made into non-immune mice no tumor growth was apparent and the animals died of virus infection in 1 to 2 weeks. When pieces of these same tumors were implanted into virus-immune mice it could be demonstrated that although the virus was present in large amounts three days after intraperitoneal inoculation an additional 3 or 4 days exposure in the intact animals was necessary before all the tumor cells were killed.

Tolerance to the Toxic Effects of Somatic Antigens of Enteric Bacilli in Typhoid and Paratyphoid Fever Convalescents. HERBERT R. MORGAN and (by invitation) FRANKLIN A. NEVA, Ann Arbor, Mich.

The somatic or O antigen (endotoxin) of *Salmonella typhosa* is the component responsible for toxicity of this organism and is a major determinant of its antigenic properties. When injected intravenously in man in minute doses, the endotoxin produces chills, fever, headache, muscle aching and generalized malaise and in some instances nausea and vomiting. Since there is evidence that the somatic antigen of *S. typhosa* is present in the blood during the acute phase of typhoid fever, it has been suggested that this toxic component might be responsible for certain of the clinical manifestations of the disease.

Following repeated intravenous injections of typhoid somatic antigen, individuals develop a resistance to its toxic effects which is not dependent on the presence of circulating O antibody which also appears. This tolerance is also protective against the similar toxic manifestations of the serologically unrelated antigen of *Shigella dysenteriae* for which no O antibodies are present.

Patients convalescent from typhoid and paratyphoid fevers and certain other infectious, febrile diseases were tested for resistance to the toxic effects of somatic antigens of *S. typhosa* and *Sh. dysenteriae*. Twenty-five of thirty-one patients convalescent from typhoid fever and eighteen of twenty-one patients convalescent from paratyphoid fever showed no significant reactions to intravenous test doses of the endotoxin of *S. typhosa* (5 γ) and *Sh. dysenteriae* (1 γ) which produce marked febrile responses and systemic reactions in normal individuals.

When retested four to six months later, eight of eleven of these patients were shown to be resistant. In the three patients who were susceptible to the initial test dose, four test injections showed that the tolerance to the toxic effects of the somatic antigens was rapidly reestablished.

Five patients, who were convalescent from other febrile infectious diseases, developed fever and systemic reactions which were comparable to those seen in normal subjects following the injection of test doses of somatic antigens.

The implications of these findings in relation to recovery from typhoid and paratyphoid fever will be discussed.

Plasma Volume, Red Cell Volume and "Extravascular Thiocyanate Space" Changes in Patients on Rice Diet. RICHARD J. F. MURPHY (Introduced by Julian M. Ruffin), Durham, N. C.

Serial observations of the plasma volume, "extravascular thiocyanate space" and red cell volume of 19 patients on the Kempner rice diet have been made. Period of observation was 14 weeks; measurements were made before starting the diet and at 3, 6, 10, and 14 week intervals following.

Seventeen patients had hypertensive disease of different types and 2 had coronary artery disease without hypertension. None had cardiac failure nor azotemia. Eleven had some alteration in renal function as measured by urinalysis, P.S.P. excretion and concentration test.

Plasma volume was measured by T1824 dilution and red cell volume was calculated from venous hematocrit.

Sixteen of the 19 patients showed significant loss of plasma volume. Mean loss in these patients was 14 per cent. This occurred chiefly during the first 6 weeks, the volume remaining relatively constant during the following 8 weeks observation.

In general loss of "extravascular thiocyanate fluid" followed the same pattern. The mean loss in this volume was 15 per cent.

Decrease in red cell volume was a constant finding in all 19 cases and showed no simple relation to fluid loss. The mean loss was 24 per cent. This could not be accounted for by blood loss from venesection for study purposes. In those cases with plasma loss only one-third

of this was reflected in the hematocrit, the other two-thirds being masked.

Weight loss was a prominent finding in some cases. There was no close correlation between weight loss and fluid loss either of degree or in time relationships. Although fluid loss was greatest in those with evident renal damage, 7 of the 8 patients with normal renal findings showed significant loss. There were no cases of frank clinical salt depletion.

There was no simple relationship between volume changes and clinical course.

Observations on the Excretion of Bromsulphalein. J. D. MYERS (Introduced by Keith S. Grimson), Durham, N. C.

Two important factors in the excretion of bromsulphalein by the liver are (a) amount of dye presented to the liver per unit of time, i.e. hepatic plasma flow, and (b) ability of hepatic cells to extract and excrete dye. Some evidence concerning the operation of these factors in normal and diseased states has been collected while measuring hepatic blood flows. Pertinent data are the arterial concentrations of BSP resulting from various dosages of dye, and the percent extraction of dye by the liver. Since the corrected infusion rate of BSP can be assumed equal to hepatic excretory rate, the data allow calculation of the BSP clearance

$$\frac{\text{infusion (excretion) rate}}{\text{arterial concentration}}$$

as ml. of plasma cleared completely of dye per minute per sq. M. Mean BSP clearance in 39 control subjects was 244 ± 83 ml.; mean per cent extraction was 53.2 ± 13.7 .

In 8 patients with severe, chronic anemia the increased hepatic plasma flow, due both to increased whole blood flow and lowered hematocrit, provided a normal if not supernormal clearance (277 ± 135 ml.) in the presence of decreased per cent extraction (30.7 ± 13.7). In 19 patients with cardiac failure, and low cardiac outputs and hepatic blood flows, the decreased clearance (89 ± 43 ml.) was due to both decreased supply of dye to the liver and impairment in hepato-cellular function (mean per cent extraction 28.5 ± 11.5). Nine individuals with Laennec's cirrhosis of moderate severity who had normal or moderately subnormal blood flows, had a markedly reduced mean clearance (134 ± 41 ml.) due largely to decreased extraction (32.8 ± 15.6 per cent). In 16 patients with hyperthyroidism mean clearance (212 ± 97 ml.) and per cent extraction (37.2 ± 17.6) were not significantly different from control values, although 6 of the group did have subnormal extraction percentages and presumably hepato-cellular dysfunction.

In both controls and ill patients, simultaneous infusion of bilirubin, human albumin, or amino-acids with bromsulphalein impaired excretion of BSP by decreasing per cent extraction.

Incidence and Nature of Hepatic Disturbance Following Acute Viral Hepatitis and in Persons with Maximal and Minimal Exposure to Hepatitis Virus: A Preliminary Report.

J. R. NEEFE and (by invitation) HUGO DUNLAP SMITH, CHARLES H. KURTZ, LEWIS WILLIAM BLUEMLE, JR., S. CLAY WILLIAMS and JOHN G. REINHOLD, Philadelphia, Pa.

The incidence and nature of hepatic disturbance has been investigated in: Group IA: 300 persons chosen at random from records containing sufficient information to justify a diagnosis of acute viral hepatitis 2 to 7 years previously; Group II (Maximal Exposure Group): 158 persons chosen at random from records indicating unusual exposure to hepatitis virus during the preceding 7 years; Group III (Minimal Exposure Group): 120 persons selected because of the lack of any recognized unusual exposure to hepatitis virus. Group IB is composed of selected patients with moderately severe symptoms and disability following acute viral hepatitis. The study of each person included a detailed history, a complete physical examination, a comprehensive group of hepatic tests, and, in representative cases, needle biopsies of the liver. An unexpectedly high incidence of findings suggesting mild hepatic disturbance has been encountered in both the maximal and minimal exposure groups. The incidence of such findings to date in the unselected post-hepatitis group (IA) has been only slightly higher. The observations on the selected post-hepatitis group (IB) support existing evidence of the occasional occurrence of a severe form of chronic liver disease with diffuse hepatic fibrosis following acute viral hepatitis. The data from the 3 "unselected" groups indicate that such major sequelae are infrequent but reveal the existence of a relatively large number of persons in all 3 groups with evidence of chronic mild hepatic disturbance which may be related to preceding apparent or inapparent infection with hepatitis virus.

Studies on Treponemal Immobilizing Antibodies Produced in Syphilitic Infection. ROBERT A. NELSON, JR., JUDITH A. DIESENDRUCK and HAROLD E. C. ZHEUTLIN (Introduced by Thomas B. Turner), Baltimore, Md.

A method has been developed by which antibodies to virulent *Treponema pallidum* can be demonstrated *in vitro*. Treponemes are extracted from rabbit testicular syphilomas and suspended in a special medium in which the organisms remain motile and infective for several days. On incubation of such suspensions with syphilitic rabbit or human sera and guinea pig complement, the treponemes become non-motile and lose their capacity to infect rabbits. This immobilizing activity is virtually absent from normal serum.

Using this technique, a preliminary survey of serum and cerebrospinal fluid from patients in various stages of syphilis has been made. Of 20 sera from patients with primary syphilis, 10 produced marked immobilization, three were doubtful, and seven showed no immobilizing effect. All sera from 20 patients with secondary syphilis, 15 with symptomatic tertiary syphilis, and two with congenital syphilis were strongly positive. All sera from 20 presumably normal individuals, and 20 patients with diseases other than syphilis were negative.

Similarly, cerebrospinal fluids from 10 patients with central nervous system syphilis were positive while fluids from 10 non-syphilitic patients were negative.

By absorption experiments it has been demonstrated that the immobilizing antibody is distinct from reagin which is detected by the ordinary serological tests for syphilis. Thus far, the sera of seven patients with unquestionable biological false-positive serological reactions were completely negative with the treponemal immobilization test.

Since the immobilizing antibody appears to be directed specifically against *T. pallidum*, it may afford a practical method for the study of the immunology of syphilis in man.

The Effects of Salt-Poor Albumin on the Excretion of Water and Electrolytes in Edematous Patients. JACK ORLOFF, LOUIS G. WELT and LYMAN STOWE (Introduced by Paul H. Laviertes), New Haven, Conn.

The infusion of 100 grams of a 25 per cent solution of salt-poor albumin may initiate a diuresis of water and sodium in edematous patients. When albumin is administered to normal subjects, however, despite similar hemodynamic changes, the excretion of sodium is decreased and that of water not significantly altered.

Albumin was injected intravenously as a 25 per cent solution into 2 patients with toxemia of pregnancy, 3 with the nephrotic syndrome, an edematous pregnant female without toxemia and 2 normal subjects. In those patients in whom the sequence of events were analyzed, there was initially a profuse water diuresis in which water was lost far in excess of sodium. The resultant elevation of the concentration of sodium in the extracellular fluid was associated with a diuresis of salt.

In contrast, in both normal individuals and in edematous patients who did not develop a water diuresis during the infusion of albumin, there was no change in the level of sodium in the extracellular water, and no subsequent increase in the excretion of salt.

From these studies it appears that the primary diuretic action of albumin in some edematous patients is to promote a diuresis of water. If water is lost sufficiently in excess of salt to elevate the concentration of sodium in the extracellular compartment, an increased excretion of sodium ensues.

Observations on Experimental Pulmonary Edema. ROBERT PAINE (by invitation) and JOHN R. SMITH, St. Louis, Mo.

Experimental heart failure in dogs with intact circulation usually causes no pulmonary edema, although pulmonary congestion may be marked. In contrast, heart-lung preparations always develop "spontaneous" pulmonary edema, in time. These phenomena were investigated in dogs. In all instances pulmonary lymph flow was measured by modifications of Drinker's technique.

In eight experiments, congestion of individual lung lobes, or of the entire lungs, produced no increase in lymph flow nor evidence of pulmonary edema.

The role of decreased plasma proteins in lung edema was investigated in nine experiments. Sufficient intravenous Locke's solution was infused to reduce the plasma proteins to about 2.6 grams per cent. Marked and sustained pulmonary lymph flow occurred, although systemic venous pressure was little influenced by the infusions. In addition, complete mechanical obstruction of the pulmonary lymphatic duct provoked no evident gross pulmonary edema; however, in the same animals Locke's infusion again resulted in lung edema.

Heart-lung preparations were set up, following isolation of the right lymphatic duct. Pulmonary lymph flow increased immediately after establishment of the heart-lung circuit (five experiments). In eleven other instances, lymph flow was carefully measured as various vessels were tied off in making heart-lung preparations. Thus, heart-lung-head preparations could be established in which the lung lymph flow was *not* increased. However, when the cephalic arteries were ligated (making an ordinary heart-lung), augmented lymph flow and eventual pulmonary edema occurred. Possibly autonomic effects from cerebral ischemia may influence the development of pulmonary edema when the heart-lung-head is transformed to the heart-lung circuit. Other evidence suggests that the rapidity of onset of pulmonary edema in the heart-lung is intensified when left ventricular overloading occurs, producing elevation of both pulmonary arterial and venous pressures, and consequent destructive rise in capillary pressure.

An Abnormality in Nitrogen Metabolism in Cushing's Syndrome Altered by Testosterone Propionate: A Study Utilizing Glycine Tagged with Isotopic Nitrogen, N 15. WILLIAM PARSON (Introduced by Thomas Findley), New Orleans, La.

Isotopic nitrogen, N 15, was incorporated in glycine and fed in single small doses to healthy men on normal and high protein diets. The rates of urinary excretion of N 15 were determined by the use of the mass spectrometer. Simultaneous nitrogen balances were made. Our results are similar to the observations of the Columbia group, reported in part by Sprinson. Approximately 30 per cent of the fed N 15 appears in the urine in 24 hours when the diet contains 1 gram of protein per kilogram of body weight. The rate increases to approximately 45 per cent on a high protein diet containing 1.5 grams of protein per kilogram.

A patient with Cushing's Syndrome in nitrogen equilibrium and maintaining her weight on a diet containing 1 gram of protein per kilogram, was given "tagged glycine" as above. N 15 excretion rates were determined. The observations were repeated during a period of nitrogen retention induced by testosterone propionate. Control observations were again made when nitrogen equilibrium returned after cessation of therapy.

In this patient, at the end of 24 hours, approximately 45 per cent of the fed N 15 appeared in the urine during both control periods. This figure fell to approximately 30 per cent during testosterone administration.

The significance of this abnormality in nitrogen metabolism is not established. If the assumption is made that "tagged glycine" traces "protein metabolism," analysis of the data suggests that the abnormality in this case of Cushing's Disease may represent "decreased protein anabolism," altered toward normal by testosterone propionate.

Cerebral Blood Flow and Oxygen Consumption in Neurosyphilis. JOHN L. PATTERSON, JR., ALBERT HEYMAN and FENWICK T. NICHOLS, JR. (Introduced by Paul B. Beeson), Atlanta, Ga.

The cerebral blood flow was determined by the nitrous oxide technique of Kety and Schmidt in 48 patients with neurosyphilis and 12 control subjects. Cerebral oxygen consumption was calculated from the flow data and the arteriovenous oxygen difference.

In patients with paresis and meningovascular syphilis the cerebral blood flow was below the normal mean in almost every instance. The mean cerebral blood flow in these conditions was 75 per cent and the lowest 32 per cent of the normal mean. The cerebral oxygen consumption was reduced in both groups, but a more marked reduction was found in the patients with paresis. In these patients the average oxygen consumption was approximately 70 per cent of the normal with an extreme value of only 24 per cent of the normal. In patients with asymptomatic neurosyphilis, both the blood flow and oxygen consumption were within the normal range.

The effect of penicillin and malarial fever therapy was studied in 17 of the patients. The mean cerebral blood flow one to six months following treatment in paresis showed no consistent change, while the oxygen consumption showed a marked mean increase (35 per cent). These changes could be correlated to a considerable degree with improvement in the patients' mental status. In most of these patients the improvement in cerebral oxygen consumption was proportionately greater than the increase in blood flow. In meningovascular syphilis the mean cerebral blood flow following therapy increased by an average of 20 per cent of its pre-treatment value, while the oxygen consumption showed only a small increase. In several patients with either paresis or meningovascular syphilis the improvement in cerebral blood flow was associated with an actual decrease in oxygen consumption or vice versa.

These studies suggest that the syphilitic process can exert a direct effect on cellular metabolism in the brain in addition to impairing its blood supply.

Postoperative Alkalosis and Potassium Deficiency. O. H. PEARSON and L. P. ELIEL (Introduced by Rulon W. Rawson), New York, N. Y.

A syndrome characterized by alkalosis, hypochloremia, and hypopotassemia has been observed in 15 patients undergoing major surgery and being maintained by intravenous feeding for several days postoperatively. Clinically these patients exhibited muscular weakness, drowsiness, nausea, abdominal distention, and cardiac irregu-

larities. Electrocardiographic changes occurred, such as depression of T waves, prolongation of the QT interval, depression of ST segments, inversion of P waves, frequent extrasystoles and auriculo-ventricular block. Administration of potassium chloride intravenously or by mouth resulted in clinical improvement, a return to normal of the acid-base and electrolyte disturbance, and disappearance of the electrocardiographic abnormalities.

These electrolyte disturbances show a striking resemblance to those observed in certain patients with Cushing's syndrome. Darrow has observed similar electrolyte changes in rats that were made potassium deficient by several different methods. We have observed the development of alkalosis and potassium deficiency in a patient treated with adrenocorticotrophic hormone. The data will be presented.

These results suggest that the pathogenesis of this syndrome in postoperative patients is due to the alarm reaction producing hyper-function of the adrenal cortex, with loss of potassium and chloride (or retention of sodium) in the urine, associated with a low intake of potassium. Other factors which may contribute to the loss of potassium and chloride will be discussed.

A Statistical Analysis of the Physiological Factors Determining Cardiac Output in Normal Subjects. RAYMOND PEARSON (Introduced by William S. McCann), Rochester, N. Y.

Heretofore, interest in cardiac output has been concerned with methods and results rather than relationships of the factors concerned. The determinants of cardiac output can be expressed by various equations relating heart rate, stroke volume, oxygen consumption, ventilation and arteriovenous oxygen difference. This study is a statistical analysis of the available data both in the literature and from our "Chest" Laboratory.

The results indicate that there is a linear increase in cardiac index from the basal state to that of severe exercise and that the rate of change seems to be fairly constant despite the method used to determine it. For each 10 per cent increase in oxygen consumption there is a 7 per cent increase in cardiac index. The arteriovenous oxygen difference shows a gradual widening with exercise but this change is minimal constituting only about 10 per cent of the total increase. Heart rate increases more than stroke volume with an increasing cardiac output. Total ventilation is directly related to either heart rate or cardiac index during exercise. As oxygen consumption increases with exercise, approximately 90 per cent of the change is accounted for by increasing ventilation whereas only 10 per cent is made up by the increase in the volumes per cent oxygen absorbed from air. The oxygen transported per heart beat reflects more of a change in arteriovenous oxygen difference than in stroke volume. Slides are presented to illustrate these changes.

Clinical Study of 11-Dehydro-17-Hydroxy-Corticosterone in Hypertension, Addison's Disease and Diabetes. GEORGE A. PERERA and (by invitation) KENNETH L.

PINES, HOWARD B. HAMILTON and KATHERINE VISLOCKY, New York, N. Y.

The present study was undertaken in order to determine the clinical and metabolic effects of synthetic 11-dehydro-17-hydroxy-corticosterone acetate (Cpd. E.) (Merck). Because of the possibility that this steroid might exert a depressor action on the arterial tension, in addition to its reported modification of carbohydrate metabolism, observations were made on four patients, two with uncomplicated hypertensive vascular disease, one with Addison's disease, and one with diabetes mellitus and hypertension.

Observations were made after an adequate baseline and on a constant regimen. Cpd. E was administered intramuscularly (20 mgs. every 6 hours) for periods usually of eight days.

At least transitory salt and water retention was observed, but an increased nitrogen excretion was not consistently apparent. Serum electrolyte values were not materially affected. A slight decrease in cholesterol esters was noted. Urinary 17-ketosteroid and "corticoid" excretion did not change significantly. Blood sugars, after 17 hours' fasting, and glucose tolerance tests were minimally altered, but the results were of doubtful significance. Transitory ketonuria appeared in some subjects. Cpd. E produced an increase in total white cell count, together with a pronounced drop in eosinophiles. Percentage uptake of radioactive iodine in the patient with Addison's disease fell slightly.

Cpd. E exerted a depressor effect on the "resting" blood pressure of the hypertensive patients.

The Use of Intravenous Human Albumin in Decompensated Cirrhosis of the Liver. JOSEPH POST and (by invitation) JEROME V. ROSE, Bronx, N. Y.

Human albumin was administered intravenously to sixteen patients critically ill with decompensated cirrhosis of the liver, for about three months, in amounts sufficient to maintain normal serum albumin levels. The dietary regime consisted of about 3,500 calories with 140 grams of protein, and supplementary B complex parenterally. Two patients had post-necrotic cirrhosis and fourteen were alcoholics with Laennec's cirrhosis. Due to the gravity of illness, the pre-treatment observation period was one to six weeks.

Of these sixteen patients, thirteen improved and had complete diureses, two showed only widening of the paracentesis intervals, and one had no effect (later died with hepatoma). Of the thirteen patients who had diureses, five died some weeks later of the following causes: "cholemia"—2; hematemesis—1; carcinoma of stomach—1; uremia—1. Seven patients who had diureses, were comatose when therapy was begun.

Six other patients, not included above, were in deep coma on admission and survived only two, two, three, seven, eight and thirteen days of therapy.

Thirty-two patients, with decompensated Laennec's cir-

rhosis of the liver, were observed during a period of six to eight weeks for control studies, while on the above dietary regime. These patients did not appear as seriously ill as were those treated with albumin. Twenty-one improved and diuresed without albumin treatment. Ten died for the following causes: "cholemia"—1; hematemesis—5; uremia—1; hepatoma—2; intestinal obstruction—1.

Intravenous albumin has a place in the therapy of decompensated cirrhosis of the liver.

The Nature of Auricular Flutter. MYRON PRINZMETAL, and (by invitation) I. C. BRILL, ELIOT CORDAY, ALVIN L. SELLERS, WALTER A. FLIEG and H. E. KRUGER, Los Angeles, Calif.

The present concept of auricular flutter, based upon the classical studies of Lewis and his coworkers, is that this arrhythmia is due to a pure circus movement moving down one auricle and up the other, ringing the venae cavae. Lewis believed that daughter waves were emitted to the periphery of the auricles from the main circus path. By taking slow motion pictures (2000 frames per second) of fluttering auricles, (250 times slower than normal speed) the flutter wave can be readily seen. When flutter is produced by placing aconitine in the center of the surface of the right auricle it can be seen that the flutter contraction travels in *all* directions from the ectopic focus and not in *one* direction as would be demanded if Lewis's circus movement were the cause of flutter. No daughter waves of contraction are visible. A burn placed across the path of the theoretical circus wave does not interfere with the flutter. Flutter contractions appear similar to the contractions in paroxysmal tachycardia. The main difference is that flutter is due to more rapid discharge from the ectopic auricular focus with varying degrees of A-V block. Auricular flutter results when an ectopic focus discharges itself at a faster rate than in paroxysmal tachycardia but slower than in auricular fibrillation. The flutter wave does not pursue a circular course but travels in all directions from the site of impulse formation by all available routes.

Differential Diagnosis of Adult Rheumatic Fever and Rheumatoid Arthritis by Means of Serological Tests.

R. W. QUINN and S. J. LIAO (Introduced by John R. Paul), New Haven, Conn.

Serological studies are reported on series of cases as follows: (i) 50 cases of active rheumatic fever; (ii) 42 cases of inactive rheumatic fever (both groups (i) and (ii) largely adults); (iii) 40 cases of acute beta hemolytic streptococcal upper respiratory infections; (iv) 35 adult cases of rheumatoid arthritis, and (v) 45 normal subjects. One to six samples of blood from each of these subjects have been studied by means of a battery of tests, including, antihyaluronidase, antistreptokinase, antistreptolysin "O," and streptococcal agglutination with both autoclaved and live organisms.

The introduction of two relatively new tests, namely (a) antihyaluronidase and (b) Thulin's agglutination technique employing autoclaved beta hemolytic streptococci, are significant points in this study. Their use places the former (a) in relation to the other streptococcal antienzyme tests and the latter (b) in relation to conventional agglutination tests using live organisms.

Results of these tests indicate that in active rheumatic fever all of the antibody titers except the agglutination titers are significantly higher than in the other groups studied. In inactive rheumatic fever titers are not significantly higher than in normal subjects, except in the case of the agglutination titers which were higher than normal. In patients convalescent from upper respiratory hemolytic streptococcal infections, the titers of all four tests are higher than in those with inactive rheumatic fever, rheumatoid arthritis or normal subjects.

Similarities in the clinical pictures of adult rheumatic fever and rheumatoid arthritis are discussed along with the serological means now at our disposal for differentiating these two conditions. This discussion indicates that in any individual case, high titers in any one or all of the anti-enzyme tests and a high agglutination titer would seem to favor a diagnosis of rheumatic fever, whereas low anti-enzyme titers and a high agglutination titer would favor a diagnosis of rheumatoid arthritis.

Study of a Human Cystinuric Fed Radioactive Methionine. LESTER J. REED, DORIANO CAVALLINI, FRED PLUM, JULIAN R. RACHELE and VINCENT DU VIGNEAUD (Introduced by Robert F. Watson), New York, N. Y.

Studies on human and experimental animals having spontaneous cystinuria have clarified some of the steps in organic sulfur metabolism. A source of urinary cystine sulfur has been proved to be ingested methionine in dogs through the use of radioactive sulfur (S^{35}). The present communication extends this type of approach to the human.

Through the incidental finding of a spontaneous cystinuria of from approximately 0.3 to 0.5 gm. per day in a patient with a post-meningitis convulsive disorder, we were provided the opportunity to study this cycle. Radioactive sulfur (S^{35}) in methionine (200 mg.) was given this patient in an amount considered safe by the standards of the Health Physics Department at Oak Ridge, Tennessee.

Following the ingestion of tagged methionine, radioactive sulfur (S^{35}) was found in the cystine of this patient's urine within 24 hours, and later was detected in the cystine of her hair. This unequivocally demonstrated that cystine sulfur may arise from ingested methionine. Coincidentally with the feeding of the S^{35} the patient had a transient increase in capillary fragility, fall in hemoglobin and decrease in red blood count. There had been an associated nutritional difficulty with the convulsive disorder. These abnormalities responded to iron therapy and the treatment of the primary clinical difficulty, and an 18 month follow-up has revealed no abnormality attributable to the radioactive sulfur.

The Action of Insulin on Glycogen Deposition in Adipose Tissue. ALBERT RENOLD and D. W. FAWCETT (by invitation) and ALEXANDER MARBLE, Boston, Mass.

The observations here reported were made during a study of lipodystrophies due to insulin. A promising line of approach was suggested by the report of Fawcett in 1948 who called attention to histological studies demonstrating under certain conditions considerable amounts of glycogen in fatty tissue.

In our studies the glycogen in the fat of diabetic rats was determined chemically and found to be negligible in untreated animals whereas amounts as large as 1.5-3.0 per cent of the fresh weight of fat could be demonstrated 24 hours after a single large dose of insulin. This action of insulin could be shown to be both local and systemic.

Young, female, alloxan-diabetic rats (40 animals) were subjected for 65 days to daily injections of 3-5 units of protamine zinc insulin into the fat pad of one groin, with control injections of saline into the other groin. The insulin-injected fat pad showed an increase in weight averaging 55 per cent, an increase in total ether-extractable material averaging 66 per cent and a definite increase in the size of individual fat cells. The ability to deposit glycogen under the influence of insulin was not consistently altered at the site of the prolonged injections.

From these data it is concluded that there is evidence of a local metabolic action of insulin in adipose tissue. This action might be attributed to the increased production of metabolically available carbohydrate—glycogen—therefore to a possible favoring action on the conversion of carbohydrate to fat in the adipose tissue itself. Considerable evidence for the existence of extrahepatic lipogenesis is available in the literature.

The Relation of Effective Pulmonary Blood Flow to Total Pulmonary Blood Flow in Normal Man and in Patients with Various Types of Chronic Pulmonary Disease. RICHARD L. RILEY, ROBERT AUSTRIAN, KENNETH W. DONALD and ANDRE COUNNAND (Introduced by Dickinson W. Richards, Jr.), New York, N. Y.

With knowledge of the composition of inspired and expired air, the respiratory quotient and the tensions of O_2 and CO_2 in systemic arterial blood, it is possible to determine indirectly the composition of alveolar air and of pulmonary capillary blood that would obtain if homogeneous equilibrium existed between blood and gas phases throughout the lung. When the composition of mixed venous blood is also determined, one can compute the proportions of mixed venous blood and of effectively oxygenated pulmonary capillary blood that, when mixed, would yield blood of the composition found in the systemic arteries. Effective pulmonary blood flow is defined as the product of total cardiac output times the calculated proportion of effectively oxygenated pulmonary capillary blood.

In normal individuals, effective pulmonary blood flow averaged 95.8 per cent of total pulmonary flow. In patients with pulmonary suppuration and in those with pul-

monary fibrosis, the effective fraction of total flow averaged 92 per cent and 88 per cent respectively. In a group of patients with severe emphysema, the average effective fraction of flow was 69 per cent, a value only slightly higher than that found in a proven case of pulmonary arteriovenous fistula.

The Summation of Penicillin and Streptomycin Activity in Vitro and in the Treatment of Subacute Bacterial Endocarditis. WILLIAM C. ROBBINS (Introduced by David P. Barr), New York, N. Y.

Despite the therapeutic effectiveness of penicillin in subacute bacterial endocarditis, ultimate failure occurs in approximately 10 per cent of all cases because of inadequate suppression of the causative organism even by massive doses. Moreover, the use of streptomycin in these cases, most of which are caused by enterococci, has effected little improvement in the results.

An investigation has been made of the combined activity of penicillin and streptomycin *in vitro* with particular reference to the quantitative relationships of their combined action on strains of enterococci isolated from patients with endocarditis. It has been observed that penicillin and streptomycin act as adjuvants in inhibiting growth of enterococci. Total summation of partial effects has been found to occur throughout a wide range of concentrations of each constituent drug. For example, complete inhibition of growth is achieved with a mixture in which each drug is present in a concentration only half that of its own inhibitory concentration.

Five patients with persistent enterococcal bacteremia, four of whom had endocarditis, have received penicillin and streptomycin concurrently in doses of moderate size. Two patients received the combined therapy terminally, but three completed a four to eight weeks course of this therapy.

In all three patients bacteremia was promptly reversed and the immediate striking clinical improvement was sustained. The apparently complete recoveries have been maintained for six to fifteen months. Two additional patients are now under treatment.

The uniformly good results in these patients has been in striking contrast to the previous general experience with the use of either drug alone, and suggests the operation *in vivo* of a summative effect comparable to that observed *in vitro*.

The Effects of Oral Therapy with Cobaltous Chloride in the Blood. JOSEPH C. ROBINSON (by invitation), ROBERT M. KARK, (by invitation) MARY MALONEY, FREDERICK SARGENT, II, and GEORGE W. JAMES, Chicago, Ill., and Richmond, Va.

Although cobalt is an effective erythropoietic agent in cobalt deficient and healthy animals, its effects on the blood of healthy adults and of patients suffering with various types of hematologic disturbances have not been clearly defined. When administered orally cobaltous chloride (20 to 240 mgs. per day) induced anorexia in some patients but no other toxic symptoms were observed.

With the above dosage schedule, increased erythropoiesis was observed in 15 patients ill with chronic sepsis, in two patients suffering with skeletal neoplastic metastases, and in one patient with an unexplained long-standing refractory anemia. No erythropoietic response was noted in patients suffering with pernicious anemia when they were fed cobaltous chloride either alone or incubated with normal gastric juice. The drug was without effect in a patient ill with nutritional macrocytic anemia.

The blood of 15 young adults ill with chronic suppurative infection, when compared with the blood of 10 bedridden controls, showed abnormal iron and copper concentrations in plasma, a decreased red cell mass and a reduced total circulating hemoglobin. Following treatment with cobaltous chloride reticulocyte responses were observed in each patient. The maximal reticulocyte percentages were usually noted between the sixth and tenth day and did not exceed 5 percent in any patient. Thereafter, a steady increase was noted in red blood cell counts, in hematocrit values and in hemoglobin levels. The plasma volume changed but slightly whereas the circulating red cell mass and the total circulating hemoglobin increased about 30 percent.

In an attempt to explain the mechanism of cobalt activity on the basis of impaired cellular respiration, the blood of patients was examined spectrophotometrically. No abnormal concentrations of methemoglobin were observed.

Tyrosine Metabolism in Human Scurvy. WALTER F. ROGERS, JR., and FRANK GARDNER (Introduced by Robert F. Pitts), Syracuse, N. Y.

It has previously been reported that scorbutic guinea pigs, and premature infants fed high protein diets, have a defect in their metabolism of tyrosine which is corrected by vitamin C. These findings have prompted the study of tyrosine metabolism in 4 individuals with scurvy and in 4 normal persons.

After suitable control periods all individuals, with the exception of one scorbutic, received 20 grams of tyrosine by mouth daily for periods varying from five to thirty-one days. The patients with scurvy, while on a vitamin C free diet, and under this load of tyrosine, excreted large amounts of "tyrosyl" derivatives, and the power of their urine to reduce phosphomolybdic acid increased markedly, presumably due to the presence of p-hydroxyphenylpyruvic acid. Compared to normal individuals receiving 20 grams of tyrosine per day, the scorbutics excreted from seven to fifteen times the quantity of "tyrosyl" derivatives, and the power of the urine of scorbutics to reduce phosphomolybdic acid was twenty to thirty times that of normals, the urine of the latter showing no increased power to reduce phosphomolybdic acid during the administration of tyrosine. The administration of vitamin C to scorbutics during tyrosine loads completely corrected the metabolic abnormality in from twenty-four to forty-eight hours.

The administration of these large amounts of tyrosine

appeared to exacerbate the clinical condition of the individuals with scurvy.

Variations in the Electrophoretic Pattern of Synovial Fluid in Articular Disease. MARIAN W. ROPES and (by invitation) DOROTHY KAUFMAN and GERTRUDE E. PERLMANN, Boston, Mass.

As reported previously, comparison of the electrophoretic patterns of synovial fluid and serum in rheumatoid arthritis supports the hypothesis that there is increased permeability to protein of the tissues interposed between the blood and the joint space. In some of the fluids the distribution of proteins followed closely that found in the corresponding serum. In other cases there was a marked elevation of gamma-globulin in the synovial fluid despite a relatively low concentration of this component in the serum.

Study of electrophoretic patterns of synovial fluids from traumatic arthritis suggests that there is differential permeability to individual protein fractions. Albumin was always higher in the fluid than in the serum, alpha-1 and beta-globulins essentially the same, and alpha-2 and gamma-globulins lower in the fluid.

Further study of rheumatoid fluids has indicated some correlation between the electrophoretic pattern of the synovial fluid, the clinical features of the involved joints and the other characteristics of the fluid. In patients with early or mild involvement of the joint studied, the electrophoretic pattern usually resembled that of traumatic fluids, with albumin higher in the fluid than in the serum and gamma-globulin in equal or lower concentration in the fluid. In cases with longer duration or greater severity of inflammation in the joint studied, the electrophoretic pattern tended to be different. The albumin concentration was equal in the fluid and serum and the gamma-globulin higher in the fluid.

The electrophoretic pattern described in the previous report, with marked elevation of the gamma-globulin in the fluid above that in the serum, was found in three cases showing atypical clinical pictures. In these patients, large effusions had been present for one to eight years, but there was relatively little objective evidence of joint damage and other studies of the synovial fluid indicated relatively mild inflammation.

The Inhibitory Effect of Secretions of the Human Respiratory Tract on Influenza Virus. HARRY M. ROSE, New York, N. Y.

Aqueous preparations of sputum from individuals with a number of diseases accompanied by cough and expectoration contain variable amounts of a substance which will inhibit the agglutination of erythrocytes by influenza virus type A (PR8) and B (Lee). This inhibitor is not specific antibody and is unrelated to the mucin content of sputum, as determined by the ACRA test, or to the level of antibodies in the blood. It acts directly on the virus and not by altering the erythrocyte.

The inhibitor is moderately heat stable but is destroyed in 30 minutes at 100° C. Its activity is essentially un-

changed over a pH range from 4.0 to 8.0. It may be partially purified and concentrated by treating sputum with chloroform, followed by precipitation with ethanol at low temperature. The partially purified material is more stable on storage than the original sputum.

The titer of inhibitor is higher against influenza virus heated to 56° C. for 30 minutes than it is against the same number of agglutinating units of living virus. This effect is more pronounced with Lee than with PR8 virus.

The inhibitor is destroyed by exposure to living PR8 virus but not by exposure to heated virus. Its activity is also moderately reduced by treatment with the R.D.E. of *Vibrio cholerae*.

Mixtures of inhibitor and PR8 virus which fail to agglutinate erythrocytes when first prepared show a progressive rise in hemagglutinin titer on standing at 37° C. This is interpreted to indicate the release of virus from an inhibitor-virus complex. Such eluted virus may or may not show a reduction in its infectivity for chick embryos.

Preparations of the inhibitor may neutralize up to 1,000 minimal infectious doses of PR8 virus in mice. Previous treatment of the inhibitor with living virus reduces its neutralizing potency.

Effect of Urethane (Ethyl Carbamate) in Multiple Myeloma. R. WAYNE RUNDLES, M. L. DILLON, EDITH S. DILLON and JOHN ARMSTRONG (Introduced by David T. Smith), Durham, N. C.

Eleven patients with multiple myeloma have been treated with urethane. One could not tolerate the chemical and three died in 1-5 weeks. Seven patients were given 120-240 grams of urethane in 8-10 weeks and the course of their disease studied over periods up to 16 months. Striking clinical benefits were observed, including subsidence of fever, relief of skeletal pain, and gain in weight and strength. Incapacitated individuals were able to resume normal activities.

Measurable improvement related to all aspects of the disease. Abnormal plasma or "myeloma" cells became fewer or disappeared from the bone marrow and showed morphologic changes indicative of retarded or arrested growth. Four individuals had severe anemia. After urethane therapy abnormal cells disappeared from the circulating blood. The hemoglobin and red cell values improved, in three cases to normal levels. There was no progression in the osteolytic skeletal lesions as judged by serial roentgenograms. Bony recalcification was demonstrable in individuals with extensive skeletal disease after 4-6 months. Serum calcium levels when elevated fell to normal. The serum proteins were studied by electrophoretic methods in five patients. After two months of urethane therapy the abnormal globulin components decreased conspicuously or virtually disappeared. The amount of Bence Jones protein in the urine decreased. There was no association between the type of serum protein abnormality or the amount of proteinuria and renal function impairment. Nitrogen retention did not develop after urethane therapy.

Incomplete remissions of the disease have been observed as well as relapses 2-6 months after discontinuing urethane. Successive courses of urethane were given with good results. Despite these reservations the therapeutic benefit of urethane in multiple myeloma exceeds that of any other agent in use at this time.

Fatal B Virus Encephalomyelitis in a Physician Working with Monkeys. ALBERT B. SABIN, Cincinnati, Ohio.

This is to report for the second time in the past 16 years that a physician, aged 25, engaged in experimental work with rhesus monkeys, died from an infection of the central nervous system due to B virus, which occurs in monkeys much like herpes simplex virus occurs in man. The first case, a fatal acute ascending myelitis, from which the B virus was isolated and identified, occurred in 1932 in a young physician who was bitten by a monkey. In the present case there was no bite, but the infection apparently resulted from contamination by monkey saliva of a minor cut on the right index finger. This was followed by an insignificant lesion at the site of the cut, lymphangitis, lymphadenitis, and within a few days thereafter by a febrile illness during which the following significant neurological manifestations were observed in the order mentioned: crops of small herpetic-like lesions on the inner aspect of the right forearm, arm, axilla and anterior chest wall associated with soreness and burning sensation, dysphagia, increasing mental confusion, absent deep tendon reflexes in lower extremities, inability to expel enema, urinary retention, bilateral nystagmus, irregular respiration and finally cessation of respiration. The most severe lesions were found in the gray matter of the posterior and lateral horns of the spinal cord, in adjacent areas of the white matter, in the regional spinal ganglia, and medulla. An infectious agent possessing the properties previously described for B virus was recovered from the affected right axillary lymph node and central nervous system, by inoculation of rabbits. Mice proved to be relatively refractory and passage was not possible in them. No virus was found in the patient's cerebrospinal fluid, liver and spleen. The virus recovered from the patient was neutralized by a specific B virus antiserum.

Unusual Serological Findings in a Group of Cases of Pneumococcal Pneumonia Associated with the Presence of Influenza Virus in the Sputum. JONAS E. SALK and (by invitation) MARY P. LYNCH, Pittsburgh, Pa.

Between October 1948 and February 1949, when influenza was not occurring epidemically in the area in which these observations were made, 73 cases of pneumonia were studied, 5 of which yielded influenza virus upon inoculation of sputum into developing chick embryos. The viruses isolated in these cases, and from an additional case of influenza-like illness, were all found to be related antigenically to the A-prime group of influenza virus Type A. *Pneumococcus* Type I was found in the sputum of 2 cases, Type II in two other cases, and Type XVI in the sputum of the fifth case. Bacteremia was present in three instances; the patients with Type I pneumonia were found

to have 1,260 and 306 colonies per cc. of blood, respectively, and one with Type I pneumonia had 11 colonies per cc.

It is of particular interest that the presence of the virus in the sputum of these patients would not have been surmised from the results of the serological studies employing the hemagglutination-inhibition test and the complement-fixation test. Even with homologous antigens, these tests failed to reveal any increase in antibody in convalescence. However, definite evidence of antibody increase was observed in one case, and suggestive evidence in another, by a method involving virus neutralization tested *in ovo*.

Although a satisfactory explanation for the vagaries in the serological findings is not immediately apparent, several plausible possibilities exist. These are at present under investigation and will be presented and discussed.

The Effects of Postural Changes, Stellate Ganglion Block, and Anemia on the Cerebral Circulation. PERITZ SCHEINBERG (Introduced by W. M. Nicholson), Durham, N. C.

Many mechanisms influencing cerebral circulation can now be reinvestigated semi-quantitatively by the nitrous oxide technique for cerebral blood flow, devised by Kety and Schmidt. This technique was modified by drawing continuous 10-minute samples of arterial and internal jugular blood, thus allowing the body to integrate the mean nitrous oxide contents rather than calculating them mathematically from 5 separate arterial and venous samples.

Effective cerebral arterial pressure was reduced by tilting to an angle of 65° from the horizontal. Unilateral release of sympathetic tone was accomplished by procaine block of the stellate ganglion. Patients with anemia had: (1) chronic blood loss of varying durations; (2) pernicious anemia.

In 13 normals the average reduction in effective mean arterial pressure on tilting was 34 per cent. Average decrease in cerebral blood flow was 21 per cent, with average increase in cerebral arterio-venous O₂ difference of 23 per cent. Cerebral O₂ utilization (cerebral blood flow \times A-V O₂) did not change. Average cerebrovascular resistance (mean arterial pressure \div cerebral blood flow) fell 16 per cent. Average fall in internal jugular venous pressure was 60 mm. H₂O.

Stellate blocks were done on 16 subjects. These fell into 3 groups: (1) normal young individuals; (2) elderly patients with diffuse vascular disease; and (3) elderly patients with acute cerebral vascular accidents. There was no significant change in cerebral blood flow, O₂ utilization, or cerebrovascular resistance following stellate blocks in any of the groups.

In 7 patients with anemia from chronic blood loss cerebral blood flows were increased greatly over the normal; the cerebral A-V O₂ differences were proportionately reduced, giving normal values for cerebral O₂ utilization. The cerebrovascular resistance was markedly diminished in all.

In 5 patients with pernicious anemia the cerebral oxygen utilization was strikingly decreased. In one the value was normal.

Renal Insufficiency Caused by Overhydration or Depression of Sodium Chloride: The "Low-Salt Syndrome."
HENRY A. SCHROEDER, St. Louis, Mo.

Under certain circumstances, especially when there is slight impairment of renal function due to the aging process, hypertension or congestive circulatory failure, oliguria leading to renal excretory insufficiency may develop rapidly. When the intake of sodium chloride is restricted and the intake of water is not, or when depression of body salt and not of water is induced, the result is nitrogen retention, dilution of extracellular electrolytes, overhydration and probably hydration of cells. Death occurs from uremia. This condition was encountered in over 30 cases, of which 10 died, under the following circumstances: (a) In congestive failure after the enthusiastic use of mercurial diuretics, (b) In congestive failure with slight renal damage when the intake of fluids was rapidly elevated, (c) In arterial hypertension with moderate renal insufficiency when the intake of salt was severely restricted, (d) Post-operatively, in older individuals, when the concentration of plasma electrolytes was diminished by too vigorous use of glucose in water, (e) When excessive electrolyte losses from surgical drainage (bile, etc.) had occurred. While the electrolyte pattern resembled that seen in anuria from other causes when fluids and not salt were given, renal function could be often restored by increasing the concentration of the electrolytes in extracellular fluids to normal levels. The intravenous injection of hypertonic saline solution (5-6 per cent), in an amount sufficient to replace the calculated deficit in extracellular fluids, was the method of choice. When this was used, diuresis often became established and renal function returned toward normal. No adverse effects from the use of hypertonic salt were observed or expected when electrolyte levels were depressed even in the presence of general anasarca, pulmonary congestion, and elevation of the venous pressure. The early recognition of oliguria due to overhydration or salt depletion is necessary for therapy to be effective. It is probable that many cases of so-called "post-operative," "reflex" and "essential" anuria are the result of overhydration. Shock was not necessary for the situation to develop.

Some Biochemical Effects of Radiation. SAMUEL SCHWARTZ (Introduced by Jay C. Davis), Minneapolis, Minn.

The effect of relatively large amounts of ionizing radiation on several biochemical systems has been studied in human subjects and in experimental animals (dogs, rabbits, and rats). The following changes have been observed:

- (1) There is a marked rise in excretion of urinary corticosteroids in human subjects.
- (2) There is a significant increase in bile pigment excretion indicating that radiation anemia is in part hemolytic in nature.

- (3) There is a marked fall in the urinary and fecal excretion of coproporphyrin.
- (4) Evidence of liver dysfunction is found shortly after the irradiation and especially during the several days preceding death.
- (5) Marked increases are often found in the urinary urochrome reaction in humans. Another tryptophane derivative, kynurenic acid, is excreted in increased amounts by dogs.
- (6) Increases are often observed in the adenosine triphosphate fraction of the circulating white blood cells. (This may be noted with relatively small amounts of radiation, but is not uniform.)

Excretion of Sodium Following Sulfanilamide Administration in Congestive Heart Failure. WILLIAM B. SCHWARTZ (Introduced by Samuel A. Levine), Boston, Mass.

It has been the hypothesis in this study that inhibition of carbonic anhydrase in the renal tubules should result in an increased excretion of sodium. Since the acidification of the urine appears to be effected by hydrogen ions within the renal tubular cells being exchanged for sodium ions in the glomerular filtrate, it was postulated that diminished tubular excretion of hydrogen should lead to a decreased reabsorption of sodium. For this reason sulfanilamide, a specific inhibitor of carbonic anhydrase, has been administered in an effort to lower the rate of formation of carbonic acid in the renal tubules and thus reduce the source of hydrogen ions available for excretion. Seven subjects in refractory congestive heart failure on constant fluid and food intakes have received sulfanilamide in doses of three to six grams per day for periods of two to eight days. Increased excretions of sodium averaging three to four times the control values have resulted in each case. There was also, in most instances, a prompt increase in potassium and water excretion and loss of weight proportional to the increased sodium excretion. At the same time there was a fall in the carbon dioxide combining power of the serum and an elevation of the serum chloride level. Significant improvement in the signs and symptoms of congestive heart failure occurred in five out of seven patients. Other compounds with unsubstituted sulfonamide groups which are inhibitors of carbonic anhydrase are under investigation in an effort to find a substance less toxic than sulfanilamide.

The Effect of Endocrine Therapy on Hormonal Excretion in Patients with Carcinoma of the Breast with Extension. ALBERT SEGALOFF and (by invitation) RICHARD L. COPPEDGE, New Orleans, La.

Studies of various hormonal excretions have been done in patients with metastatic carcinoma of the breast. Many of the patients showed levels of gonadotrophic hormone excretion much lower than one would expect from their age and endocrine status. This is believed due to their advanced cachectic state as were the low ketosteroid values observed in many of these same patients. In the patients in whom gonadotrophic hormones were sufficiently high before the initiation of therapy, both estrogens and andro-

gens produced the expected decrease of gonadotrophic hormone in the urine. Most of the patients receiving testosterone propionate showed the expected rise in ketosteroid excretion and androgen excretion. However, two of the patients with previously low ketosteroid values had no increase in ketosteroid excretion.

In 3 of the 6 patients in whom follow-up cortins were obtained, there was a very distinct rise in the excretion of cortin while on testosterone therapy. In the one patient under estrogen therapy in whom this has been studied, there was no change in the excretion of cortin in the urine despite a favorable clinical response to the estrogen.

In 3 of the 4 patients in whom the urinary excretion of lactogenic hormone has been studied before and during therapy with testosterone, there was a distinct rise in prolactin excretion during the therapy with testosterone. In one of these 3 patients, estrogen produced no further change.

There has been no very striking correlation between the clinical course observed in any of these patients and the hormonal changes in the urine. However, the patients obtaining the best clinical responses to testosterone were the patients who showed the increases in cortin excretion.

The Metabolism and Excretion of Electrolytes and Glucose in Diabetic Acidosis. DONALD W. SELDIN and ROBERT TARAIL (Introduced by David M. Kydd), New Haven, Conn.

Fifteen patients, studied during the early stages of diabetic acidosis, received repeated moderate or massive injections of glucose. Utilization and storage of carbohydrate probably occurred within the first two to three hours of treatment, since the injected glucose could not be completely recovered in the extracellular fluid or urine. The large increment of administered glucose which was excreted after massive injections resulted in a striking increase in urine flow despite dehydration. Potassium was not swept into the urine by the osmotic action of glucose within the renal tubule, since the excretion of the ion was highest initially and fell progressively notwithstanding marked increases in glycosuria. The excretion of sodium and chloride, in contrast, appeared to be augmented by infusions of glucose, although this response was modified by adaptive reactions such as conservation of sodium in the maintenance of acid-base equilibrium.

The serum concentrations and urinary losses of potassium and phosphorus were highest initially, and fell progressively during treatment. The high serum concentrations resulted from the failure of the initially accelerated excretion to keep pace with the effects of rapid cellular release of the ions and a contracting extracellular volume. After treatment was instituted, however, the discharge of potassium and phosphorus from cells was progressively retarded. Indeed, positive intracellular balances of phosphorus were observed in six of seven patients and of potassium in two of eight patients. The slow cellular release of the ions (possibly due to increased utilization of carbohydrate and diminished catabolism of protein), in conjunction with continuing losses in the urine and expansion

of extracellular volume were responsible for the fall in the serum concentrations.

The Effect of Lumbodorsal Sympathectomy upon Insulin Tolerance in Man. F. A. SIMEONE (Introduced by Oliver Cope), Boston, Mass.

The insulin tolerance of non-diabetic hypertensive patients selected for lumbodorsal sympathectomy did not differ from that of non-diabetic non-hypertensive patients of the same age group. In the cat, dog, and rabbit, denervation of the adrenal glands renders the animals extraordinarily sensitive to insulin. In a previous report, it was demonstrated that, contrary to expectation, the insulin tolerance of patients who had had lumbodorsal sympathectomy was increased during the immediate postoperative period. This finding was interpreted as a manifestation of the adaptation syndrome.

Twenty-six patients have been studied approximately one year after lumbodorsal sympathectomy and the insulin tolerance curves have been compared with those before operation and with those immediately after operation in the same patients. The sensitivity to insulin a year after operation was significantly greater than it was before operation or soon after operation.

In three of the twenty-six patients, the increased sensitivity to insulin a year after operation was especially striking, the blood sugar concentration remaining 20 per cent below fasting as long as 180 minutes after the injection of insulin. One of these three had a convulsion after insulin, 0.08 units per kilogram of body weight intravenously. No other patient had a convulsion before or after operation.

In summary, lumbodorsal sympathectomy is attended by increased sensitivity to insulin demonstrable after a preliminary period of increased tolerance, a manifestation of the adaptation syndrome. In the majority of cases, however, the change in sensitivity is not great enough to be a useful test of adrenal denervation for the individual patient.

The Site of Action of Methyltestosterone in the Production of Creatinuria. ETHAN A. H. SIMS (Introduced by Arthur J. Geiger), New Haven, Conn.

It is generally accepted that methyltestosterone ingestion produces increased creatine synthesis, which is followed by creatinuria when the capacity to store creatine is exceeded. The initial rise in guanidoacetic acid (GAA) serum levels and excretion indicates accelerated GAA synthesis. It has not, however, been demonstrated that creatinuria may be produced solely by increased GAA synthesis. Failure to recover quantitatively exogenous creatine or GAA in balance studies, including a recent study in which the subject's tissue creatine was labeled with N-15, suggested that the methylation of GAA is retarded by excess creatine. If this were so, it would be necessary to postulate that methyltestosterone accelerates the methylation as well as the synthesis of GAA.

GAA tolerance tests made after the ingestion of 25 gm. of creatine gave results inconsistent with a retarded con-

version of GAA to creatine under these conditions. To determine whether GAA is in fact methylated when the tissues are loaded with creatine, a normal subject was given 2.5 gm. of GAA daily in 16 hourly portions after the body had been saturated by prolonged creatine ingestion. A creatinuria averaging 0.48 gm. was seen during the following 21 days. The creatinine excretion did not fall during this period, suggesting that the urinary creatine did not originate from body creatine. All creatine values were corrected for glycoxyamidine. GAA excretion averaged 0.55 gm. per day during the period of creatinuria. No evidence suggesting retardation of methylation of GAA was found under these conditions. It was thus possible to simulate the creatinuria from methyltestosterone by administering GAA in physiological amounts. This suggests that the direct action of the steroid on creatine metabolism may be solely that of accelerating GAA synthesis.

The Dynamics of the Cellular Response of Lymph Nodes to Acute Bacterial Infection. RALPH O. SMITH (by invitation) and W. BARRY WOOD, JR., St. Louis, Mo.

In order to define more precisely the role of lymphatic tissue in the cellular mechanism of antibacterial defense, a study was made of the pathogenesis of acute popliteal lymphadenitis produced in rats by inoculation of the footpad with Type I pneumococcus. Early in the course of the infection the bacteria reaching the popliteal node via lymphatics caused a rapid exudation of polymorphonuclear leucocytes within the nodal sinuses. The granulocytes which accumulated in the intermediary sinuses were found to come primarily from capillary blood vessels of the node, whereas the majority of inflammatory cells in the subcapsular sinus arose from the primary focus of infection in the foot. The granulocytes in the sinuses, carried toward the hilus by the continuous flow of lymph, formed a "log jam" of cells through which invading bacteria were forced to pass. The hilar accumulation of cells greatly increased the filtration efficiency of the node, and pneumococci caught among the phagocytes were promptly ingested. Mast cells lining the sinuses were noted to discharge their heparin containing granules into the nodal lymph. Whether this mast cell reaction accounts for the failure of lymph within the node to clot or whether the released heparin plays some other role in antibacterial defense is now being investigated. It is concluded that lymph nodes, often regarded as passive filters in the lymph stream, in reality react promptly to bacterial invasion and provide an active cellular barrier to bacterial invasion of the blood stream.

Liver Function Studies in Amebiasis. W. A. SOBEMAN and (by invitation) A. A. DOERNER, New Orleans, La.

Liver function tests (cephalin flocculation, thymol turbidity, quantitative blood bilirubin, bromsulphalein) were done in a group of 51 patients with colonic amebiasis in the absence of dysentery, clinical evidences of malnutrition and past history of hepatic disease. They fell into four groups: (1) Acute amebic abscess, (2) Acute amebic

hepatitis without demonstrable abscess, (3) Colonic amebiasis with tender hepatomegaly but without systemic response, and (4) Colonic amebiasis without demonstrable hepatomegaly. Tests were done before therapy was instituted and, where possible with abnormalities, after therapy was completed.

Approximately 50 per cent of those in groups 1 and 2 showed evidence of hepatic damage, chiefly with the cephalin flocculation test and with minor elevations of the blood bilirubin. In 10 per cent there was clinical jaundice. In group 3 the tests were positive in about 20 per cent and, like group 4, were spotty and irregular.

These studies indicate that (1) in the absence of a febrile response, the variability of results was not striking. (2) Although diffuse damage occurs in the liver from amebiasis and is reflected in liver function tests, the process at times may reach advanced stages with no more evidence of involvement, by the technics used, than in those with subclinical involvement. (3) The number of such discrepancies between the clinical findings and the liver function tests used is in contrast with that seen in some other acute conditions of the liver and may be helpful at times in differential diagnosis. (4) Moderate degrees of hepatomegaly, unexplained and without confirmatory positive tests of liver function, are at times the chief physical findings of amebiasis and should lead to search of the colon for this disease. (5) Amebiasis may, at times, without clinical signs of hepatic involvement give evidence of hepatic involvement with the liver function tests used.

Coronary Blood Flow and Cardiac Oxygen Consumption in Unanesthetized Dogs. F. C. SPENCER, S. R. POWERS, D. L. MERRILL and R. J. BING (Introduced by Cowles Andrus), Baltimore, Md.

In a large series of experiments on trained, unanesthetized dogs, the coronary blood flow through the left ventricle has been determined by the combination of coronary sinus catheterization and the nitrous oxide method. A catheter was inserted into the coronary sinus through the external jugular or cephalic vein; arterial blood was collected from the femoral artery. Following a ten-minute saturation with 15 per cent nitrogen oxide, samples were collected over a desaturation period of eight minutes. Mixed venous blood was collected simultaneously through a separate catheter in the pulmonary artery. Left ventricular weight was calculated from the tables of Herrmann.

The left ventricular flow averaged 151 cc./100 grams/minute, the coronary arterio-venous oxygen difference 12.8 vol. per cent, and the oxygen consumption of left ventricular muscle 19.5 cc./100 gram/minute. The oxygen consumption of the whole left ventricle averaged 11.3 cc. of oxygen/minute. The left ventricular efficiency

$$\left[\frac{\text{left ventricular work (kgm. meter-s)}}{\text{energy cost of whole left ventricle (kgm. meter-s)}} \right]$$

calculated from an average cardiac output of 4,450 cc./minutes and a mean aortic pressure of 119 mm. Hg was found to be 30.7 per cent.

It may be concluded that in the unanesthetized dog the left ventricular flow and oxygen consumption per unit

weight are twice as high as in the anesthetized dog (Edenhoff *et al*). Accordingly the left ventricle efficiency is within the range observed in normal man.

Metabolic Effects of Synthetic Compound E (17-Hydroxy-11-Dehydrocorticosterone) in 2 Patients with Addison's Disease and in 1 with Coexisting Addison's Disease and Diabetes Mellitus. RANDALL G. SPRAGUE and (by invitation) MARSCHELLE H. POWER, HAROLD L. MASON and HARLEY E. CLUNTON, Rochester, Minn.

Response to the administration of synthetic compound E (17-hydroxy-11-dehydrocorticosterone) was studied in 2 female patients with Addison's disease and in 1 with both Addison's disease and diabetes mellitus during treatment with 11-desoxycorticosterone. Compound E acetate was administered intramuscularly as an aqueous suspension of finely ground crystals in doses of 50 and 100 mg. daily, respectively, to the 2 patients with Addison's disease, and in doses of 30 mg. to the patient with both Addison's disease and diabetes. In another study, the last patient received 25 mg. of free compound E daily.

Under the conditions of these studies, compound E produced no significant change in the urinary excretion of sodium, potassium or chloride. Likewise, minor changes in the excretion of nitrogen, calcium and phosphorus were probably not significant.

During treatment with compound E, both patients with Addison's disease were able to fast without decline of the level of the blood sugar. A minor reduction in sensitivity to insulin, and higher levels of the blood sugar in the course of glucose tolerance tests were also observed. In the case of coexisting Addison's disease and diabetes mellitus, carbohydrate effects of the hormone were more easily demonstrated. During treatment with compound E, there was a marked increase in urinary glucose, in spite of the daily administration of 4 or more times the amount of insulin employed in the control periods.

During and following periods of administration of compound E acetate, the urinary 17-ketosteroids and corticosteroids increased above the control levels. In 1 patient, the excretion of these substances remained elevated for a period of four weeks after the administration of compound E acetate was discontinued, and there was evidence of persistence of its physiologic effects during this time, suggesting that the steroid was absorbed slowly from the sites of injection.

The results demonstrate the predominant carbohydrate activity of compound E. The hormone was excreted in part as a 17-ketosteroid.

Cardiovascular-renal Dynamics and Electrolyte Excretion in Panhypopituitarism. J. STAMLER, A. P. FISHMAN and R. ROSENMAN (Introduced by L. N. Katz), Chicago, Ill.

Increased appreciation that hormonal factors influencing mineral exchange may play a role in the initiation and persistence of congestive heart failure led us to study the cardiovascular-renal dynamic changes in various endocrine disorders usually associated with electrolyte disturbances. This report presents the initial data in this investigation.

In two cases of panhypopituitarism, with B.M.R.'s of -48 and -23 respectively, studies of the cardiovascular-renal dynamic changes by cardiac catheterization and renal clearance techniques revealed the following changes: decreased cardiac output, cardiac index, circulation rate, renal blood flow, renal index (renal blood flow/cardiac output), glomerular filtration rate, and blood volume, and normal thiocyanate space and venous pressure.

Patient A presented symptoms suggestive of adrenal insufficiency (plasma Cl 90, Na 134 m. eq./L); patient B had no such findings (plasma Cl 102, Na 144 m. eq./L). With distal tubular function calculated on the basis (H. W. Smith) that there is an obligatory isotonic proximal tubular reabsorption of 85 per cent of the glomerular filtrate, renal clearance data reveal the following: In patient A, with plasma Cl concentration of 89-91 m. eq./L, 6.7-7.4 per cent of the Cl filtered (GFR: 47 cc./min.) is excreted; the overall tubular reabsorbate has a Cl concentration of 86-87 m. eq./L; distal tubular Cl reabsorption is at the rate of 0.3-0.4 m. eq./min.; the distal reabsorbate has a Cl concentration of 58-62 m. eq./L. In patient B, despite a higher plasma Cl concentration (94-104 m. eq./L) and despite a higher GFR (58 cc./min.) only 2.0-4.2 per cent of the Cl filtered is excreted; the overall tubular reabsorbate has a higher Cl concentration (102-114 m. eq./L); distal tubular Cl reabsorption occurs at a greater rate (0.7-0.8 m. eq./min.); by further contrast, the distal Cl reabsorbate is hypertonic (267-485 m. eq./L), and this cannot be accounted for by impaired H₂O reabsorption. Thus, the inadequacy of distal electrolyte absorption in patient A, even at low plasma Cl levels, contrasts markedly with that of patient B and accounts for the hypochloremia and hyponatremia in the former.

It is suggested that patient A, unlike patient B, is one of the more unusual cases of panhypopituitarism suffering from a secondary adrenal insufficiency involving the mineralocorticoids. In such a patient both the decreased demands upon the heart (low B.M.R.) and the decreased tubular reabsorption of electrolytes secondary to adrenal insufficiency mitigate against the development of congestive heart failure and edema, despite myocardial damage and low glomerular filtration rate (decreased presentation of Na to the renal tubules).

The Effect of the Iodide Ion on Thyroid Hormone Formation in Man. MALCOLM M. STANLEY (Introduced by E. B. Astwood), Boston, Mass.

The amount of iodine entering the thyroid normally provides an index of hormone formation. Stable thyroid iodine (I^{127}) accumulation can be estimated with radioactive I^{131} provided (1) That thyroid I^{131} can be measured and (2) That the specific activity of the collected I^{131} can be computed. $I^{127} = I^{131}/S.A.$

Nearly simultaneously, serially, urine I^{127} and I^{131} , serum I^{127} and thyroid I^{131} were determined, the last by counting at 35 centimeters from the gland. The kidneys excreted these isotopes in the urine in the same ratio in which they appeared in the serum: $SI^{127}/SI^{131} = UI^{127}/UI^{131}$. The renal and thyroid arterial blood iodide contents were identical; the urinary $I^{131}:I^{127}$ ratio reflected the specific activ-

ity of the thyroid radioiodide accumulated during the period. Serum iodide (I^{127}) = $SI^{127} \times UI^{127} / UI^{131}$.

The thyroids of these twenty controls with serum iodide levels around one microgram or less (without extra iodide) accumulated 5-16 micrograms I^{127} /hour, averaging 11 micrograms/hour. As serum iodide levels were raised to 6-12 micrograms per cent by iodide administration the iodine accumulated by the thyroid proportionally increased. With higher serum iodide levels inhibition of organic binding of iodide in the thyroid occurred, with subsequent complete blocking of uptake. This extended the observations of Wolff and Chaikoff in the rat. The thyroids in eight thyrotoxic patients accumulated an average of 122 micrograms I^{127} /hour; inhibitory serum iodide levels were less than five micrograms per cent.

With varying serum iodide values below inhibition the thyroid collects a constant fraction of the serum iodide. Probably the amount of iodide in the thyroid cells, rather than in the serum, determines the inhibitory point; with great ability to accumulate iodide (high thyroid: serum ratios) inhibition occurs with low serum iodide levels, and vice versa.

Fat in Intravenous Nutrition. FREDERICK J. STARE and (by invitation) SHERWOOD W. GORENS and ROBERT P. GEYER, Boston, Mass.

The inability to supply caloric requirements is a handicap in present-day parenteral therapy.

Work on the preparation of fat emulsions for intravenous use and on some of the metabolic aspects of the intravenous administration of fat has been under way in this laboratory. In the past year fat emulsions have been given to patients with varying degrees of success. A few cases will be mentioned of which the following is typical.

The patient was a 24-year-old white male with onset of frequent bloody mucous stools 7 years ago. A diagnosis of ulcerative colitis was made. Fifteen months prior to admission, he began to have 9 to 12 bowel movements per day and he lost 45 pounds. An ileostomy was performed. The ileostomy did not function properly, and the patient developed a peritoneal infection and a mid-jejunal fistula. It was necessary to reoperate to close the fistula. At this time the patient weighed only 90 pounds and was in an extremely poor state of nutrition. Oral intake was nil, and the usual parenteral therapy contributed no more than 400 calories per day. On the first postoperative day 300 ml. of a 15 per cent fat emulsion containing 1.6 calories/cc. were given; on the second day twice this amount was given. The patient showed marked clinical improvement and felt much stronger. For the next 10 days he received daily infusions of fat varying from 600 to 900 ml. and furnishing 960 to 1,440 calories. By this time oral intake of food was sufficient and all parenteral therapy could be terminated.

Summarizing, fat emulsions can be prepared which can be given intravenously without reaction, and which contribute appreciably to caloric intake without requiring large fluid volumes or prolonged infusion times.

The Serum Proteins in Portal Cirrhosis—Electrophoretic Studies. Effects of Therapy. KENNETH STERLING and WILLIAM E. RICKETS (by invitation), and JOSEPH B. KIRSNER and WALTER L. PALMER, Chicago, Ill.

Electrophoretic analyses of the serum proteins and liver function tests were performed on ten normal subjects and twenty patients with proven portal cirrhosis of the liver, with follow-up studies during the course of medical management in five patients.

The principal abnormalities observed in the serum proteins of 14 untreated patients were diminutions of the albumin fractions and elevations of the gamma-globulin fractions. Less frequently observed and usually less pronounced were elevations of the beta-globulin fractions. Of these 14 cases, seven with ascites and hyperbilirubinemia exhibited these alterations in marked degree, so that the gamma-globulin fractions usually approached or even exceeded the albumins. Seven patients without ascites manifested a considerable range of variation in the electrophoretic pattern from markedly abnormal to nearly normal in three cases without symptoms.

Serial electrophoretic studies and tests of hepatic function were done on five patients with ascites and hyperbilirubinemia during the course of medical management for nine months or less. Progressive change in the direction of normal coinciding with clinical improvement was observed during the administration of a high protein, high carbohydrate, high caloric diet. Infusions of plasma and concentrated human albumin were followed by pronounced and immediate elevations of the serum albumin values.

Six patients who had clinically recovered from severe portal cirrhosis and had been asymptomatic for more than two years were found to have electrophoretic patterns very close to normal.

These studies indicate that the alterations in the serum protein pattern in portal cirrhosis are not irreversible, but with prolonged medical management and clinical recovery, may revert practically to normal.

Alcohol and Homeostasis: The Uncompensated Water Diuresis Induced by Whiskey. MAURICE B. STRAUSS and (by invitation) JACK D. ROSENBAUM and WILLIAM P. NELSON, III, Framingham, Mass.

Although the diuretic effect of strong drink was commented upon by W. Shakespeare, modern scientific opinion has leaned toward the thesis that it is the large quantity of fluid so often simultaneously imbibed which provokes the increased urinary output. The occurrence of undue thirst following the ingestion of strong or even much diluted alcohol appears to be a matter of common knowledge albeit little concerning this may be found in scientific literature.

Five hours after breakfast, volunteers ingested 147 cc. of Bourbon whiskey or water. Following the former, the urine output was increased three-fold or more, but the excretion of strong electrolyte exhibited no significant deviation from the values observed after water.

In order to compensate for insensible water loss in the fasting, thirsting state, the urine should contain at least

a third more electrolyte than the body fluids if hypertonicity of the latter is to be avoided. Since, after whiskey, the urine was observed to contain as little as one-fourth the electrolyte of body water and to be excreted in rather large volume, it is apparent that whiskey defeats the wisdom of the body and significantly disturbs homeostasis.

The rather constant glomerular filtration rate observed indicates that whiskey diuresis results from decreased tubular absorption of water. Evidence suggests that this is not due to a direct effect of whiskey on the tubules but rather an effect mediated through the supra-optico-hypophyseal mechanism.

No evidence was obtained of any alteration in the distribution of water between cells and extra-cellular fluid except that which would be anticipated as a result of the induced osmotic changes.

Both in the control and the whiskey studies the excretion of strong electrolyte often exhibited three-fold variation in spite of the fact that the amount of filtered electrolyte remained constant or varied in the opposite direction. Glomerular filtration of strong electrolyte would appear to be of less significance in controlling the rate of electrolyte excretion than has recently been suggested.

Studies of Formation, Diffusion and Absorption of Constituents of Cerebrospinal Fluid in Man. I. Studies with Na²⁴. W. H. SWEET, B. SELVERSTONE, A. SOLOMON and L. BAKAY (Introduced by James C. White), Boston, Mass.

The rate of entrance of normal constituents into cerebrospinal fluid (CSF) from blood is being studied in man, with minimal disturbance of the hydrodynamic system. Needles placed in one lateral ventricle, cisterna magna and lumbar subarachnoid space remain in position throughout the experiment. Following intravenous injection of 16 microcuries of Na²⁴ per kilogram, we withdraw at frequent intervals, samples of CSF not exceeding 0.2 cc. from each of the three points with concurrent blood samples. The final CSF pressure is not significantly altered from the initial level. Radioactive assay of specimens permits the drawing of satisfactory curves describing the rate of disappearance of Na²⁴ from blood serum and its rate of appearance in CSF at each of the three points sampled.

Studies have been carried out not only on individuals in whom the circulation of CSF was presumably normal, but also in patients with "hypertensive meningeal hydrops," and in patients in whom a recent cerebral lobectomy for neoplasm had been carried out. Studies have been repeated after subsidence of the acute process in several patients.

A striking feature of the results has been the rapidity of appearance of Na²⁴ in the lateral ventricle as contrasted with a slower rate in the cistern and an extremely slow rate in the lumbar subarachnoid space. In both the normal and the abnormal individuals the concentration of Na²⁴ in the ventricle was three to four times that in the cistern at one hour, with an even greater differential prior to this time. In addition there appears to be an unexpectedly sluggish rate of flow and diffusion from the ventricles to

the cisterna magna, since a number of hours elapse before the concentrations at the two points become the same.

A Proplasin Activator from Mammalian Tissue. H. J. TAGNON and (by invitation) MARY L. PETERMANN, New York, N. Y.

The proteolytic enzyme plasmin is present in normal blood plasma as an inactive precursor called proplasmin. Activation can be brought about by treatment of plasma with certain organic solvents or by the action of streptokinase. In certain pathological conditions, for instance traumatic shock, the active enzyme may appear in the circulating blood. This results in digestion of fibrinogen and fibrin and may lead to uncontrollable hemorrhage. The mechanism of activation of proplasmin in shock is unknown.

The experimental evidence presented here indicates that an activator of proplasmin is present in tissue, notably in lung. By differential centrifugation in the cold, it was possible to separate from an aqueous extract a particulate fraction possessing most of the activity of the extract while an inhibitor which was present in the extract remained in the supernate, and could thus be separated from the activator.

The activator was not removed from the particles by several washings. The activator transformed proplasmin into plasmin even in the presence of the plasmin inhibitor of plasma. Optimal pH range extended from 6.0 to 7.2. The temperature optimum was 37° C. The amount of plasmin produced appeared to be proportional to the amount of activator and independent of the quantity of substrate (proplasmin). The activator lost its activity by incubation at 37° C. for 4 hours or at 56° C. for ½ hour. It could be preserved indefinitely in the frozen state.

The presence of a proplasmin activator, probably a kinase, in tissue may explain the fibrinolysis in exudates and the resorption of blood clots from tissues. Whether such an activator will provide an explanation for the fibrinolysis of shock remains to be demonstrated.

The Effect of Sodium Withdrawal upon the Body Weight of Normal Young Men. CAROLINE BEDELL THOMAS and (by invitation) EVELYN HOWARD and ARLENE ISAACS, Baltimore, Md.

A disturbance of salt and water metabolism in hypertensive patients has been described by Perera and Blood. They reported that, following 24 hours of sodium restriction, the elimination of salt and water from the body as reflected in loss of body weight was much less marked in patients with hypertension than in normal subjects. Their work raises two questions:

1. In a larger group of normal subjects, do some respond to sodium withdrawal like hypertensive patients?
2. If such individuals be found, is there any evidence that they will eventually have hypertension?

The body weights of 83 male medical students were observed following 24 hours of sodium restriction (sodium chloride 1 gram) while on a standard diet. Among 64 un-

selected students, a loss of 1.4 per cent of total body weight was the most frequent change observed. 47 lost 0.9 per cent or more, while 17 lost less than this amount, and in this respect resembled the hypertensive patients. Duplicate tests were carried out on 24 subjects, including 15 subjects showing the "hypertensive pattern" of response; results were consistent in 79 per cent of cases. A considerable proportion of normal young men, therefore, respond to sodium withdrawal with the "hypertensive" metabolic pattern.

A number of the unselected subjects had a history of hypertension affecting one parent, high normal resting blood pressure levels, a positive cold pressor test, or were overweight, and others selected because of these factors were included in the study. The correlation between these possible "prehypertensive" factors and the inability to lose weight following sodium withdrawal is discussed.

Correlation of Clinical Manifestations of Infection with the Elaboration of Hyaluronidase by Pathogenic Pneumococci. ROBERT T. THOMPSON and FRANCES E. MOSES (Introduced by Morton Hamburger), Cincinnati, Ohio.

Pneumococci isolated from the eighteen to twenty-four hour blood cultures of thirty patients were tested for the ability to elaborate hyaluronidase by inoculation into 0.20 per cent hyaluronic acid broth. Hyaluronidase elaboration was determined by the mucoprotein clot prevention test. Twenty-one patients had primary pneumococcal pneumonia; nine others had pneumonia associated with some other disease. Cultures from ten primary pneumonia patients failed to elaborate hyaluronidase; one of these patients died. Cultures from eleven primary pneumonia patients elaborated hyaluronidase; three of these patients died. Considering all thirty cases, the duration of infection before blood culture seemed related to hyaluronidase elaboration by the infecting pneumococci. Of the cultures from five patients who had been ill only one day, one elaborated hyaluronidase and four did not. Of the cultures from eighteen patients who had been ill two to five days, nine elaborated hyaluronidase and nine did not. Of the cultures from seven patients who had been ill for seven days or longer, six elaborated hyaluronidase and one did not.

In the twenty-one cases of primary pneumococcal pneumonia, there was no relation between the age of the patients or the number of lobes involved and hyaluronidase elaboration by the infecting pneumococci. However, of the cultures from eleven patients with blood urea nitrogen under 40 mgm. per cent, four elaborated hyaluronidase and seven did not. Of the cultures of eight patients with blood urea nitrogen above 40 mgm. per cent, five elaborated hyaluronidase and three did not. The blood urea nitrogen was not determined in two patients.

These findings indicate that pathogenic pneumococci are more likely to elaborate hyaluronidase in the infections of longer duration. In primary pneumococcal pneumonia hyaluronidase elaboration by infecting pneumococci is often associated with elevation of the patients' blood urea nitrogen.

The Influence of Streptomycin Dosage on the Pattern of Emergence of Drug Resistant Tubercle Bacilli in Patients with Pulmonary Tuberculosis. RALPH TOMPSETT (Introduced by Walsh McDermott), New York, N. Y.

Demerec has shown that the development of streptomycin resistance of staphylococci *in vitro* proceeds by a series of stepwise increases in the degree of drug resistance of the bacterial population. In contrast with the homogeneity of steps in penicillin resistance, the first-step streptomycin resistant strains as well as those of subsequent steps contained some organisms of the highest degree of resistance. It is manifestly impossible to eliminate these highly resistant bacteria by the highest concentrations of streptomycin attainable *in vivo*. Nevertheless, it is conceivable that elimination of intermediate steps might effect a significant reduction in bacterial population during the time required for the highest step resistant strains to assume predominance, and hence prolong the effective period of drug action. It seemed of interest, therefore, to determine if a similar stepwise pattern of resistance is demonstrable *in vivo*, and the influence of streptomycin dosage on its appearance.

Observations have been made on strains of tubercle bacilli obtained from sixty-four patients with pulmonary tuberculosis treated with streptomycin. In 33 patients who received 3.0 gm. of streptomycin daily, the streptomycin resistance of all but one of the strains of tubercle bacilli fell into two well-defined steps. All strains were highly sensitive to streptomycin (2.0 micrograms per ml.) prior to treatment. Strains obtained subsequently were found to remain at this level or to exhibit such a high degree of resistance that they were not inhibited by 100 micrograms per ml. of streptomycin.

In contrast, a high proportion of strains obtained from 31 patients given only 1.0 gm. of streptomycin daily exhibited degrees of resistance intermediate between these two extremes. As many as half of the strains obtained during certain periods were found to be streptomycin resistant to various degrees less than the maximum.

Effect of the Intravenous Injection of Vaccinia Virus on the Survival of Mice with a Transmissible Leukemia. JOSEPH C. TURNER and (by invitation) BARBARA MULLIKEN, New York, N. Y.

In the course of investigations designed to examine the influence of extraneous viruses on experimental neoplasms, it appears that the behavior of lymphatic leukemia 9417 in inbred mice may be modified by the introduction into the hosts of certain strains of living vaccinia virus. In five separate experiments extending over a period of some months, of 148 untreated control animals 13 (9%) survived for 30 days after the inoculation of leukemia and only 3 (2%) for 60 days or more. In the same experiments 187 mice received single or multiple intravenous injections of suspensions of vaccinia at varying intervals after the transplantation of the leukemia. Of these, 49 (26%) lived 30 days and 28 (15%) survived 60 days or more.

The variables involved are numerous and have not been fully resolved. The magnitude of the effect has so far appeared to be inversely proportional both to the acuteness of the leukemia and, perhaps paradoxically, to the virulence of the strains of virus. Thus, in the experiments noted above, vaccinia of low virulence, prepared from mouse-brain culture or frozen pulp (calf), were used. In other experiments highly virulent virus from rabbit-brain or egg-membrane have completely failed to influence the mortality of leukemic mice.

The mechanism of the observed effect remains uncertain; nor has it yet been settled whether the effect is determined by the infective particles themselves or some soluble component of the tissue suspension. Despite the difficulties attending the evaluation of findings in experiments with transmissible tumors, the point may be made that, as judged from an unequivocal end-point, survival, the behavior of a highly malignant neoplasm appears to have been modified in consequence of the introduction of a biological agent by the intravenous route at some time after the tumor may be assumed to have become established.

The Inheritance of Facioscapulohumeral Progressive Muscular Dystrophy; Variability in Expression in Human Inheritance as an Explanation of So-called "Anticipation." FRANK H. TYLER (Introduced by G. E. Cartwright), Salt Lake City, Utah.

A study has been made of a large kindred, certain members of which have facioscapulohumeral dystrophy. There are 1,249 individuals in six generations in the established pedigree. The inheritance is typical of a Mendelian dominant trait with complete penetrance and variable expressivity. Among the completely or nearly completely studied sibships which represent simple back crosses ($Dd \times dd$) there are 130 dystrophies and 143 normal individuals. The deviation from the expected 1:1 ratio is insignificant.

The disorder in the average patient is only slightly incapacitating. However, there is great variability in the severity of its expression in middle and old age. Even the patients with the more severe disabilities of the disease have had large families. This fact has permitted an enlightening comparison of the severity of the disease in the offspring and in the parent. This has shown that in certain families the children were more severely involved than the parents. In other families, the parents show the most marked changes.

In families in which diabetes mellitus or myotonia dystrophica occur, it has long been assumed that the disorder becomes progressively more severe in successive generations. This phenomenon has been called *anticipation* or *antedating*. Another explanation for the observed variation in these diseases is variability in the expression of the trait and the fact that in severe cases, beginning in adolescence, procreation becomes highly improbable. Comparison with the findings in facioscapulohumeral dystrophy where large families can be studied, suggests that this is the true explanation rather than "anticipation."

The Use of Emission Spectrography in the Study of Metabolism of Metals in the Blood in Man. BERT L. VALLEE and JOHN R. LOOFBOUROW (Introduced by M. D. Altschule), Boston, Mass.

Emission Spectrography is based on the principle that high energy excitation of atoms causes them to emit light of characteristic wavelengths; each element has a typical spectral pattern which may be recorded photographically. This technique permits the simultaneous identification of practically all elements present in a given sample. Quantities as low as fractions of one microgram may be detected accurately. Quantitation of at least five or six elements simultaneously is possible.

Previous use of this technique resulted in identification of Zinc in leucocytes. Now in progress are studies of the distribution of elements in the fluid and corpuscular components of human blood. Studies have been made on Magnesium, Manganese, Copper, Strontium, Rubidium, Titanium and other metals regularly found in blood. Some of these elements, heretofore, have not been thought to be of biological significance.

The results of the present investigation are of interest in view of the known relation of metals to various enzyme systems present in erythrocytes and leucocytes. In some instances, the element is an integral part of the enzyme molecule such as in the case of iron in catalase, cytochromes, veridoperoxidase, zinc in carbonic anhydrase and copper in tyrosinase. In others, in vitro activation by various metallic ions has been found essential as exemplified by arginase, phosphatase and succinic dehydrogenase.

The significance of our findings in regard to the metal enzyme relationships will be discussed.

The Iron Metabolism and Acid Fragility of Erythrocytes in Paroxysmal Nocturnal Hemoglobinuria. PHILIP F. WAGLEY and MARGARET ADAMS (by invitation), and JOSEPH F. ROSS, Boston, Mass.

Two subjects (L. G. and L. T.) with paroxysmal nocturnal hemoglobinuria were given radioactive iron by parenteral injection and samples of their blood were taken at subsequent intervals. For a time the maximum possible age of the erythrocytes containing radioactive iron would correspond to the interval between the injection and the sampling. Washed erythrocytes derived from aliquots of blood taken at specified intervals after the injection of radioactive iron were subjected to hydrochloric acid hemolysis at a pH of 6.7-6.9 and determinations were made of the relative amounts of hemoglobin and of radioactive iron released. During a short period following injection, acid hemolysis released two to three times more radioactive iron from the red cell mass than could be accounted for by release of hemoglobin.

Most of this radioactive iron released by acid hemolysis was not loosely bound ("easily splittable iron"); 73-96 per cent of the radioactivity was precipitated with the proteins by trichloroacetic acid.

These observations have been considered in light of two possibilities: (1) the susceptibility of the red blood

cell to acid hemolysis may vary with age of the cell, or (2) all of the radioactive iron released by acid hemolysis is not in hemoglobin, some being in other protein bound form(s). The implications of these possibilities will be discussed.

The Effect of Auricular Fibrillation on the Coronary Circulation. RENÉ WÉGRIA and (by invitation) RICHARD P. KEATING, HENRY P. WARD and CHARLES W. FRANK, New York, N. Y.

Forty-five bouts of auricular fibrillation induced in twelve dogs were studied as to their effect on the mean arterial blood pressure and coronary flow.

At the onset of auricular fibrillation, a marked drop in blood pressure and coronary flow occurs. Then, within a few seconds, both blood pressure and coronary flow return toward their control levels, but whereas the coronary flow may, at least temporarily, return to or even rise above its control level, the blood pressure very seldom reaches it. As soon as fibrillation stops, both coronary flow and blood pressure rise above control levels, then come down progressively to their control level, but the blood pressure may come back to its control level before the coronary flow. The mechanisms and significance of the cardiovascular reactions observed will be discussed.

Growth Forms of Common Bacteria Indistinguishable from Pleuropneumonia-Like Organisms. HOWARD J. WEINBERGER and LOUIS DIENES (Introduced by J. H. Means), Boston, Mass.

The authors will present additional evidence indicating that many commonly occurring bacteria can survive and multiply in a peculiar form not recognizable by the usual bacteriological methods.

Freshly isolated strains of many bacillary species transferred to appropriate media containing varying concentrations of penicillin undergo a peculiar transformation which results in the development of colonies indistinguishable from those of the Pleuropneumonia group. Strains of Typhoid, Salmonella, Shigella, H. influenzae, Proteus, Bacteroides, Flavobacterium, Streptobacillus moniliformis, and a Gram positive spore bearing bacillus exhibited this phenomenon. In Salmonella, Proteus, Bacteroides and Streptobacillus these Pleuropneumonia-like (L) forms can be made to revert to the usual bacillary forms indicating that they are growth forms of the bacteria despite the marked differences in their morphology, staining reactions, physical properties, and growth and reproductive characteristics.

The effect of penicillin is not specific. Similar transformation occurs under other "untoward" influences including autolysis of the cultures. Exposure of typhoid bacilli to fresh human serum also has resulted in the development of typical L colonies.

The Pleuropneumonia group is responsible for important animal diseases. In humans Pleuropneumonia-like organisms have been recovered from prostatic, urethral and vaginal secretions, bladder and kidney urine, joint fluid, empyema fluid, paronychia and peritoneal abscesses and sputum.

The nature of these organisms is not fully understood and in most cases the relation of the organisms isolated from pathological processes to a parent form has not been established. The pathological significance of bacteria in the Pleuropneumonia-like form warrants intensive study. Such organisms have been observed under conditions similar to those prevailing in the body. In light of the widespread therapeutic use of penicillin and the frequent incorporation of penicillin in culture media, it is possible that the incidence of Pleuropneumonia-like organisms will increase.

Studies of the Influence of Penicillin on the Immune Reactions in Streptococcal Pharyngitis. LOUIS WEINSTEIN and (by invitation) LOUIS BACHRACH and THOMAS S. PERRIN, Boston, Mass.

A group of 225 patients with pharyngitis due to the beta-hemolytic streptococcus was treated with penicillin given in a dose of 15,000 units intramuscularly every 3 hours for 10 days. Serial examinations of their serums for antistreptolysin, antistreptokinase, anti-M, and anti-C substance antibody and the "phase" reacting material of Coburn, at weekly intervals for a month or more revealed that there was a marked reduction in the ability to produce all of these antibodies. In contrast to the 75-85 per cent incidence of antistreptolysin and the 25-35 per cent incidence of antistreptokinase in control untreated patients, of those given penicillin only about 12 per cent developed significant antistreptolysin and/or antistreptokinase titers. In the treated individuals who showed an increase in either or both of these antibodies, the height of the titer attained was considerably lower than in the average control patient. None of the treated group developed any anti-M or anti-C substance antibody and the "phase" reactions indicated the lack of value of this test as a diagnostic aid in rheumatic fever. About 7 per cent of the treated individuals developed rheumatic fever while approximately 6 per cent showed evidence of a varying degree of glomerular nephritis; of these patients, only about $\frac{1}{3}$ had a rise in antistreptolysin or antistreptokinase in the blood.

When the penicillin treatment schedule was changed to administration every 8 hours either intramuscularly or orally, the number of individuals showing significant antistreptolysin titers increased remarkably. Thirty-two per cent of 103 patients given 100,000 units intramuscularly every 8 hours showed an increase in this antibody. Exhibition of penicillin by mouth on an 8-hour schedule showed an even lesser tendency to inhibit antibody formation and in a group of 241 patients given the drug in doses varying from 50,000 to 800,000 units, from 49 to 80 per cent showed an increase in antistreptolysin. These differences in antibody formation could not be correlated with persistence or recurrence of streptococcal infection since the vast majority of the patients were rid of their organisms in 24-48 hours after therapy was instituted. That the increased incidence of antibody formation may be due to longer persistence of Streptococcus in the pharynx is suggested, however, in the group receiving 500,000 units of penicillin orally every 8 hours since, in these pa-

tients, recurrence of the organisms in the pharynx was common after treatment was stopped. A small number of individuals receiving oral penicillin showed a rise in the anti-M substance antibody.

The Fate of Transfused Leukocytes. AUSTIN S. WEISBERGER (by invitation), ROBERT W. HEINLE and (by invitation) JOHN P. STORAASLI and RICHARD HANNAH, Cleveland, Ohio.

Elevation of the leukocyte count following whole blood transfusions does not occur even in severely leukopenic recipients. A study of this phenomenon was undertaken. Concentrated suspensions of leukocytes were obtained from the peritoneal cavity of rabbits by distending their abdomens with physiologic saline. If radioactive phosphorus (P^{32}) was administered previously in doses of 1 millicurie, the cells obtained were radioactive. The fate of such transfused cells was studied by determining the radioactivity of tissues of recipient rabbits.

The peritoneal fluid was collected, centrifuged and washed. Practically all of the P^{32} was fixed in the cells. These were resuspended in Tyrodes solution and transfused into the same or other rabbits.

Following the intravenous injection of radioactive or non-radioactive leukocytes, an initial increase of blood leukocytes never occurred. Instead, a profound leukopenia developed usually within 1 to 5 minutes. In more than half the animals a subsequent leukocytosis occurred after 4 to 6 hours. In 5 rabbits sacrificed during the leukopenic stage, the distribution of radioactivity was: lung, 45.4 per cent; liver, 20.7 per cent; spleen, 1.0 per cent. In marked contrast, the distribution following injection of inorganic P^{32} was: lung, 1.3 per cent; liver, 11.9 per cent; spleen, 0.33 per cent. Following intra-aortic injection of radioactive leukocytes into 5 rabbits, the distribution of radioactivity was: lung, 30.5 per cent; liver, 17.8 per cent; spleen, 0.35 per cent. Following disappearance of the leukopenia (4 to 6 hours), the distribution in 5 rabbits was: lungs, 19.1 per cent; liver, 34.9 per cent; spleen, 3.4 per cent.

These findings suggest that the lung plays an active role in controlling the blood leukocyte level. Since cell-free aqueous extracts of leukocytes produce a similar profound leukopenia, the arrest of leukocytes in the lungs represents more than a simple filtering of foreign bodies.

Effects of Infusion of Albumin on the Excretion of Water and Electrolytes in Normal Subjects. LOUIS G. WELT and JACK ORLOFF (Introduced by John P. Peters), New Haven, Conn.

During infusions into normal subjects of 100 grams of a 25 per cent solution of salt-poor albumin the rate of excretion of sodium decreases. There is no significant alteration in water excretion. These changes are associated with an increase in plasma volume. They are not correlated with the degree of change in total protein or albumin concentrations in the serum nor with a significant change in endogenous creatinine clearance.

Administration of a 10 per cent solution of albumin resulted in an increase in plasma volume and a definite de-

crease in the rate of excretion of sodium and water. In contrast, when albumin was administered as a 4 per cent solution water excretion rose strikingly and sodium excretion questionably. This reaction was less marked when a 5 per cent solution was administered.

It appears that when albumin is infused in normal subjects in concentrations with a higher colloid osmotic pressure than that of serum the rate of water and sodium excretion diminishes below that obtained with iso-osmotic albumin infusions. When the plasma volume is expanded, but the colloid osmotic pressure remains normal the kidneys respond in such a manner as to correct over-hydration; but when expansion of plasma volume is accompanied by excessive colloid osmotic pressure their response is adjusted to correct dehydration. Gradations of these reactions elicited by varying the concentrations of injected albumin suggest that the responses are controlled by the balanced action of two forces, colloid osmotic pressure and plasma volume.

It is also possible that a decrease in interstitial fluid volume in normals initiates a mechanism that increases the reabsorption of sodium and water; and an increase in plasma volume unassociated with a decrease in interstitial fluid volume provokes a diuresis.

A Comparison in the Same Individual of the Nitrogen Loss Following Surgery with that Produced before Operation by an Identical Feeding Program. SIDNEY C. WERNER, New York, N. Y.

Twenty-eight patients on approximately adequate diets in terms of calories and protein have been given a reduced diet similar to that usually given post-operatively, both before and after the procedure. The resultant alterations in nitrogen balance have been compared. No significant increase in negative nitrogen balance occurred after cholecystectomy, inguinal or extensive ventral hernia repair, provided constant feeding was maintained through the operative day and subsequently.

The data obtained suggest that most of the work during the war concerning the effect of trauma or disease on nitrogen balance cannot be interpreted as indicating the role of injury. First, the preceding dietary intake was often not known. Second, a constant intake of calories and protein was not maintained from the moment of trauma. Third, the increased demands for calories resulting from fever have not been taken into account, in most studies.

Since no changes in nitrogen balance after operation have been found which could not be accounted for on the basis of inadequate calories, a review of the typhoid fever work on "toxic destruction of proteins" has been made. Here too, adequate provision of calories produced nitrogen equilibrium at the height of disease, a finding similar to the war period findings in this and other laboratories on the effect of increased protein, or calorie and protein, intake after injury or operation. The one study suggesting that an excess of calories is needed in typhoid fever to restore nitrogen equilibrium is vitiated by the fact that the patients lost weight on a theoretically adequate diet.

It is thus suggested that the "catabolic response" or "toxic destruction of proteins" is probably the result of

lack of provision of adequate calories, protein, or both, and not of unusual body mechanisms being brought into play; or if such effects are present, that, divorced from under-nutrition, they are not highly significant.

Antidiuresis and Renal Vasoconstriction Following Venous Congestion of the Limbs in Normal, Hypertensive, and Splanchnicectomized Subjects. ROBERT W. WILKINS, (by invitation) JAMES W. CULBERTSON, BELTON A. BURROWS, CLARENCE M. TINSLEY and WALTER E. JUDSON, and CHARLES H. BURNETT, Boston, Mass.

When the legs of supine subjects were congested by blood pressure cuffs inflated on the thighs at less than diastolic pressure for 10 to 20 minutes, there was usually little change in arterial pressure or pulse rate, and little effect on renal circulation or function until after the cuffs were released. Ten to twenty minutes after the release of the congesting cuffs there was a marked antidiuresis (as much as 90 per cent), a decrease (25 to 60 per cent) in renal plasma flow (PAH) and filtration rate (inulin, mannitol), and a reduction of sodium, potassium and chloride excretion (as much as 60 per cent). In 10 to 20 minutes the changes in renal circulation and electrolyte excretion had usually disappeared, but the antidiuresis persisted for at least 40 minutes.

Similar renal functional and circulatory effects followed venous congestion of the limbs of hypertensive subjects before and after lumbodorsal sympathectomy.

These results appear significant in connection with the various mechanisms of water and salt retention and the disturbances of renal circulation in venous congestive myocardial failure. They indicate that venous congestion of the limbs, and possibly of other peripheral areas, may contribute to the causes of reduced renal plasma flow and excretion of water and salt in this condition. Apparently these renal disturbances may persist for some time after the relief of venous congestion. They are not necessarily preceded by changes in renal arterial or venous pressure, and are not mediated over the splanchnic sympathetic nervous system since they are present after splanchnicectomy. Possibly they are mediated through humoral mechanisms.

The Application of a Strain Gauge Manometer, a Modified Ear Oximeter and Whole Blood Cuvette Oximeter to the Technic of Cardiac Catheterization. EARL H. WOOD, EDWARD LAMBERT and HOWARD B. BURCHELL. (Introduced by Charles F. Code), Rochester, Minn.

A strain gauge manometer (Statham Model P6-250-15D) coupled without amplification to sensitive galvanometers affords one of the most accurate and simple means of recording pressure through a cardiac catheter. Uniform sensitivity to sine wave variations in pressure of from 0 to 28 cycles per second may be obtained through a cardiac catheter if proper electrical and mechanical damping are used. This is superior to the dynamic response obtained with a Hamilton manometer (natural frequency: 120 cycles per second). The strain gauge manometer is connected to (1) a visual indicating galvanometer utiliz-

ing a red light thus permitting continuous monitoring of pressures during roentgenoscopy and (2) an oscillographic galvanometer located in an adjacent recording room which permits simultaneous and continuous photographic recordings of the variations in pressure transmitted through the catheter. It is recognized, however, that the physical characteristics of the catheter and the pressures generated within its lumen by the acceleration and deceleration of the fluid column associated with the movements of the catheter imparted by the heart beat obviate the possibility of obtaining uniformly reliable tracings of intracardiac pressure with manometers attached to the external end of the catheter.

Variations in arterial saturation are recorded continuously on the oscillograph and monitored simultaneously from a visual reading scale by means of a modified ear oximeter.

The oxygen saturations of samples of blood withdrawn from the catheter are recorded and monitored similarly with a whole blood cuvette oximeter. The electrocardiogram and respiration are recorded simultaneously.

Immediate correlation of oxygen saturation of samples of blood withdrawn from the catheter with associated observations of pressure, arterial saturation, and location of the tip of the catheter (roentgenoscopy) greatly expedites the procedure. The probable diagnosis was apparent prior to withdrawal of the catheter in the majority of cases in which analysis of all data ultimately proved sufficient for such a decision to be made.

The Chemoprophylaxis of Scrub Typhus in Volunteers. T. E. WOODWARD and H. L. LEY JR. (by invitation), and J. E. SMADEL, Washington, D. C.

Two field trials were conducted in Malaya in which 75 volunteers were exposed in hyperendemic areas of scrub typhus. Thirty-four of these individuals received Chloromycetin prophylactically for a total period of three weeks during and following exposure. They did not show clinical evidence of scrub typhus throughout the period of prophylaxis or the ensuing week. However, scrub typhus developed in 15 volunteers in the prophylactic groups of the two trials 5 to 14 days after drug was discontinued. Although the attack rate among the volunteers in the two field trials varied markedly, there was no essential difference in the ultimate infection rates among the controls and those given prophylaxis in each test.

Scrub typhus, when it developed among volunteers in the prophylactic group, was not significantly different from the disease in the controls except for the absence of eschar formation.

Relapses were prominent features of the disease in the volunteers of both prophylactic and control groups. These had not been observed previously in untreated cases of scrub typhus or in naturally occurring cases which were treated with chloromycetin. Fifty-four per cent of the 37 persons in the two trials who contracted scrub typhus suffered one or more relapses. Various factors probably contributed to this phenomenon.

Prolonged administration of chloromycetin as a prophylactic measure and its use in the treatment of the disease

attacks of scrub typhus as well as the relapses indicated that the drug is of low toxicity for man, and that drug-fast strains of *R. tsutsugamushi* are not readily produced.

Experimental Production of Hemolytic Disease in Newborn Dogs. LAWRENCE E. YOUNG and (by invitation) DONALD M. ERVIN, RICHARD M. CHRISTIAN and R. WENDELL DAVIS, Rochester, N. Y.

Isohemagglutinins of 4 different specificities have been demonstrated in sera of dogs following transfusions of dog erythrocytes containing antigenic factors lacking in their own cells. Since the behavior of one of these antibodies is in some respects similar to that of Rh antibodies, dog antisera having this specificity have been tentatively designated "anti-Do," and dog cells have been classified as "Do-positive" or "Do-negative" depending upon their reaction with such sera.

Hemolytic disease has been produced in Do-positive puppies born to a Do-negative dam immunized by transfusions of Do-positive cells before mating with a Do-positive sire.

In the first litter investigated, erythrocytes of 4 affected Do-positive whelps were agglutinated by high dilutions of anti-dog-serum rabbit serum and were also agglutinated when suspended in undiluted normal dog serum, presumably because the cells were coated with Do-antibody. Plasma from 4 unaffected Do-negative whelps in the same litter agglutinated the sire's cells, but iso-antibodies were not detectable in the plasma of the affected Do-positive puppies. The dam's breast milk agglutinated the sire's erythrocytes but absorption of iso-antibody from milk by the puppies could not be demonstrated.

The degree of anemia and icterus varied markedly among the affected whelps of the same litter. Erythrocytes of all of the Do-positive puppies, however, showed increased osmotic fragility, and reticulocytes and nucleated red cells were more numerous in venous blood of the affected puppies. Autopsy of a severely anemic whelp and of a normal litter mate revealed more marked erythropoiesis in liver, spleen and marrow of the affected animal.

Observations thus far completed suggest that studies on hemolytic disease in newborn dogs may aid in clarifying certain aspects of the comparable human disorder, and that controlled investigations on large litters sired by heterozygous males may be especially productive.

Intrathoracic Pressure Change as an Aid in the Anatomic Localization of Murmurs and Other Sounds of Cardiovascular Origin. HARRY F. ZINSSER, JR. (by invitation) and CALVIN F. KAY, Philadelphia, Pa.

Certain commonly used diagnostic techniques are based on the relationship of murmur intensity to rapidity and volume of blood flow. By exercise, amyl nitrite and other means, circulatory velocity is increased to accentuate murmurs. If blood flow could be altered *differentially* in the lesser and greater circulations, the time of murmur accentuation should provide a clue to its anatomic origin. Such changes in circulatory dynamics occur in the relaxation period immediately following voluntary straining.

During straining, venous blood return is decreased. The right heart output falls, the pulmonary reservoir is depleted, and systemic output falls. When intrapulmonic pressure returns to atmospheric level, venous blood rushes first into the right heart, causing vigorous pulmonary artery pulsations and restoring the lesser circulation. Left heart output and aortic pulsations become maximal only after the pulmonary blood reservoir is restored.

It was predicted, therefore, that murmurs should become faint during straining and gain intensity after relaxation. Murmurs of right heart or pulmonic origin should become intense immediately in the post-straining period, while return of left sided or peripheral murmurs should be delayed.

Studies were undertaken with combined phonocardiographic, electrokymographic, and electrocardiographic techniques upon subjects with murmurs of either known or unknown origin. Variations in intrathoracic pressure produced by straining against a mercury manometer were recorded with strain-gauge attachment.

Recordings bear out the predicted results. Murmurs were loudest either early, associated with maximal electrokymographic pulmonary artery pulsations; or late, coinciding with maximal aortic pulsations. Certain patients fail to produce effective straining. Where straining was effective, the method successfully differentiates known right sided from left sided murmurs. In patients with murmurs of unproven type, equally decisive results seem significant by deduction. Alterations of other cardiac sounds also occur, similarly suggesting the site of production.

THE ELIMINATION OF PENICILLIN G IN BILATERALLY NEPHRECTOMIZED DOGS^{1, 2}

BY WILLIAM H. ANDERSON³ AND ROLF BRODERSEN⁴

(From the Department of Medicine, University of Chicago)

Rammelkamp and Keefer (1) found that only about 60 per cent of intravenously administered penicillin could be recovered from the urine of man. This finding has been confirmed by later reports, although the recovered amounts vary from 40 to 99 per cent (2).

This paper deals with the mechanism of the elimination of penicillin in bilaterally nephrectomized dogs.

Diffusion of Penicillin from Blood to Tissues

A dog, in which the elimination of penicillin⁵ had been blocked by bilateral nephrectomy and ligation of the cystic and common bile ducts, was given an intravenous injection of 25,000 units/kg. of crystalline penicillin G. Blood samples were taken from another vein at intervals from two minutes to 10 hours after the injection. The serum concentrations of penicillin were determined using a serial dilution technique.

In Figure 1, the serum penicillin concentrations have been plotted on a logarithmic scale as a function of time after the injection. The penicillin concentration decreases rapidly during the first hour but thereafter very slowly. For the time being we shall consider only the first portion of the curve. We may assume that this initial decrease was due to diffusion of penicillin from the blood stream into the tissues.

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³ Research Fellow, U. S. Public Health Service.

⁴ Douglas Smith Foundation Fellow in Medicine.

⁵ The crystalline penicillin was supplied by Abbott Laboratories, Commercial Solvents Corporation, Lederle Laboratories, and Schenley Laboratories, Inc.

One can imagine this process as taking place through a thin membrane from one phase to another. At the time $t=0$, all penicillin is in the one phase having the concentration c_0 . If the concentration in this phase is c_t at the time t and c_∞ when equilibrium is established we have:

$$\ln (c_t - c_\infty) = \ln (c_0 - c_\infty) - Kt \frac{c_0}{c_0 - c_\infty}, \quad (1)$$

where \ln denotes the natural logarithm and K is the velocity constant.

From the data in Figure 1, K is found to be 5 1/hours. The curve in the figure is drawn according to equation (1), this value being used for K and allowance being made for the inactivation in the later part of the experiment. Agreement is found between the calculated curve and the experimental points. The value, 5 1/hours, for K was confirmed in another experiment of similar nature as the one depicted in Figure 1.

If there were no further elimination of penicillin from the blood, the concentration would decrease to a constant value which would be reached when diffusion equilibrium was established. This concentration may be found by drawing the straight line representing the inactivation back to intersection with the axis of the ordinate which, in this case, was 143 units/ml. of serum.

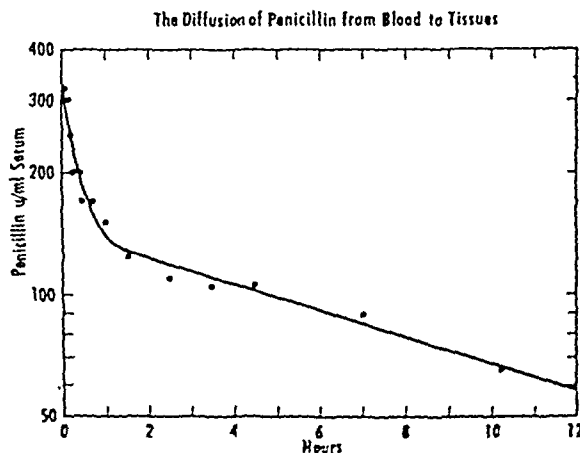


FIG. 1. THE SERUM PENICILLIN CONCENTRATION AS A FUNCTION OF THE TIME AFTER A SINGLE INTRAVENOUS INJECTION

The inactivation has been partially blocked by bilateral nephrectomy and ligation of the cystic and common bile ducts.

The equilibrium concentration was determined in a number of experiments and collected in Table I. In the last column of this table are the ratios between c (serum concentration of penicillin at equilibrium) and the dose of penicillin per gram of dog. In 16 experiments this ratio was found to be larger than unity; that is, the penicillin did not distribute equally throughout the tissues of the dog; but was present in a higher concentration in the plasma than in the remainder of the dog. The average ratio was about four times as great, when diffusion equilibrium was established. The reason for this is not clear.

TABLE I

The serum concentration (c_{∞}) of penicillin after diffusion equilibrium has been established with the tissues

Values are corrected for any elimination or inactivation of penicillin taking place.

Dose units/kg.	c_{∞} units/ml.	c_{∞} units/ml. dose units/gram of dog
85,000	400	4.7
80,000	250	3.1
30,000	130	4.3
25,000	90	3.6
25,000	70	2.8
25,000	80	3.2
25,000	100	4.0
25,000	85	3.4
25,000	100	4.0
25,000	110	4.4
25,000	80	3.2
25,000	100	4.0
25,000	140	5.6
14,000	60	4.3
14,000	60	4.3
7,000	20	2.9
Average		3.9
Maximum		5.6
Minimum		2.8

From Table I we obtain the formula:

$$\text{Serum penicillin concentration} = 4 \times \text{amount of penicillin per gram body weight. (2)}$$

—provided that diffusion equilibrium is present. This equation shows the relationship between the amount of penicillin present in the body and the serum concentration. This could be used to estimate the former when the latter is known. The formula has been established for the blood concentrations between 20 and 400 units/ml. and may not be valid for smaller concentrations.

The Renal and Extra-Renal Elimination

Blood serum concentrations of penicillin were determined at intervals after a single intravenous injection in a number of normal dogs. If allowance is made for the diffusion equilibrium to be established, it was found that the logarithm of the

concentration decreased as a linear function of time (first order reaction). This relationship was found to be valid from the initial concentration of about 100 units/ml. to 1 unit/ml. A curve demonstrating this relationship is shown in Figure 2.

Bilateral nephrectomy was performed in three dogs which were then given a single intravenous injection of penicillin. The relationship between serum concentration and time showed that these dogs were still able to eliminate penicillin from the blood stream, although, as was to be expected, at a considerably lower rate (Figure 2). Here also, the inactivation took place as a reaction of the first order. The rate of elimination was, as an average, found to be one-fourth as rapid in the nephrectomized as in the normal dogs. This means that approximately three-fourths of the penicillin was eliminated through the kidneys and one-fourth somewhere else. From the variable velocities obtained in normal and nephrectomized dogs, a considerable variation is to be expected in the fraction of penicillin inactivated extra-renally.

The Site of the Extra-Renal Elimination

The rate of elimination was not significantly changed by the removal of the intestine from the

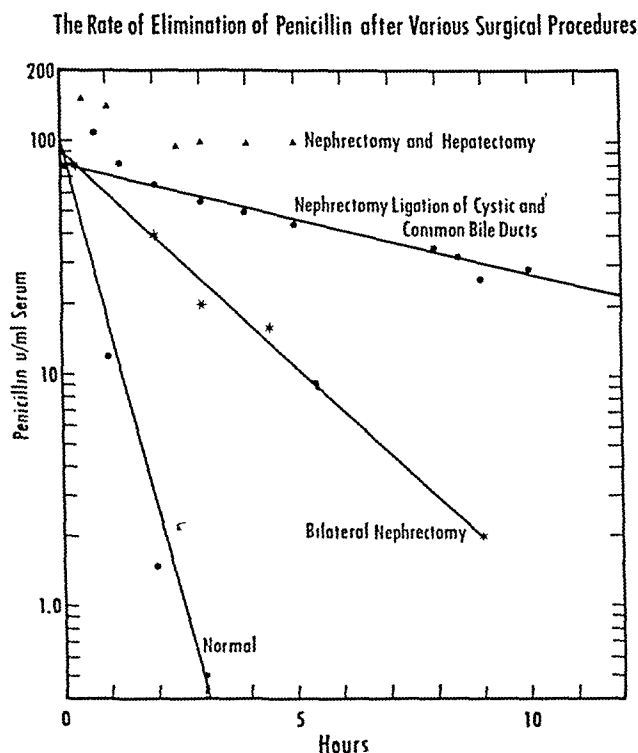


FIG. 2. ALL DOGS WERE GIVEN 25,000 UNITS OF PENICILLIN PER KILOGRAM IN A SINGLE INTRAVENOUS INJECTION

ligament of Treitz to within an inch of the anus, nor by the removal of the stomach and ligation of the common bile duct. This showed that, in these dogs, the intestinal tract was not the primary site of the extra-renal elimination, as suggested by the findings of Reid (3).

The serum penicillin concentration remained constant, within the limits of experimental error, following complete evisceration; thus the site of the elimination was somewhere in the viscera.

A two stage hepatectomy and bilateral nephrectomy were performed in a dog. Here too, no inactivation could be shown after the equilibration of penicillin between the blood stream and the tissues. In another dog, both kidneys and all the viscera except the liver were removed. The hepatic artery was left intact by dissecting away the hepatico-duodenal ligament. The portal vein and the common bile duct were ligated and the gall bladder left in connection with the biliary system. This dog eliminated penicillin at a rate very similar to the dogs in which only bilateral nephrectomy was done. It could be concluded that the liver was responsible for the extra-renal elimination of penicillin in these dogs.

The velocity constants were found from the obtained curves by the formula:

$$K = -\frac{\ln(c_{t_1}/c_{t_2})}{t_1 - t_2} \quad (3)$$

The results, in reciprocal hours, are found in Table II. The velocity constant indicates the relative decrease in penicillin concentration, or total amount of penicillin, per hour.

The Mechanism of the Hepatic Elimination of Penicillin

In two dogs the kidneys were removed, the cystic and common bile ducts were ligated twice and cut. Similar curves were found in both dogs; one curve is shown in Figure 3. It was noted that a certain elimination of penicillin seemed to occur during the first 10 hours; however, the rate of elimination decreased after this to a very low value. Thus, penicillin was still present in appreciable amounts 70 hours after the intravenous injection. The final rate of elimination here was only 4 per cent of the normal rate. This finding seemed to indicate that the penicillin was excreted in the bile rather than destroyed by the liver; if

TABLE II

The velocity constant indicating the relative decrease in penicillin concentration in the serum after various surgical procedures

Procedure	K 1/hrs.	Average
Normal dogs	1.85	1.70
	1.90	
	2.40	
	1.90	
	1.25	
	1.15	
Nephrectomized	1.45	0.41
	0.46	
	0.38	
Nephrectomized and the intestine re-moved from the ligament of Treitz to within an inch of the anus	0.40	0.34
Nephrectomized, bile duct ligated and complete gastrectomy	0.35	
Eviscerated, except for the liver	0.53	
Complete evisceration	0.0	
Hepatectomy, nephrectomy	0.0	
Cystic and bile ducts ligated; nephrectomized	0.07	
Bile duct cannulated—excreted in bile	0.31	
	0.25	
Inactivation in bile at $-\log \text{cH} + 8.5$, 40°C .	0.10	

The Slow Elimination after Bilateral Nephrectomy and Ligation of Cystic and Common Bile Ducts

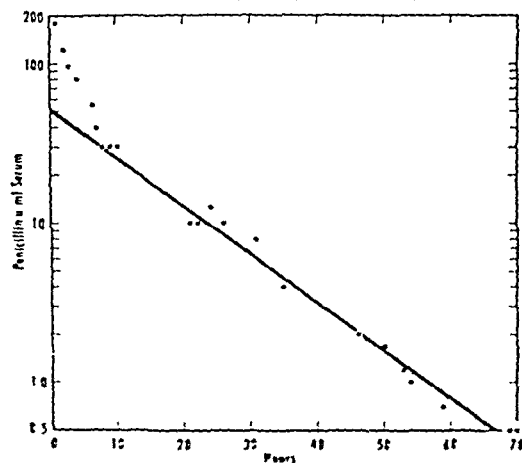


FIG. 3. THE ELIMINATION DURING THE FIRST 10 HOURS MAY BE DUE TO EXCRETION OF PENICILLIN CONTAINING BILE INTO THE BILIARY SYSTEM TO THE POINT OF DISTENTION

The slower inactivation throughout the 70 hours of the experiment can be explained by the destruction of penicillin due to the alkalinity of the bile.

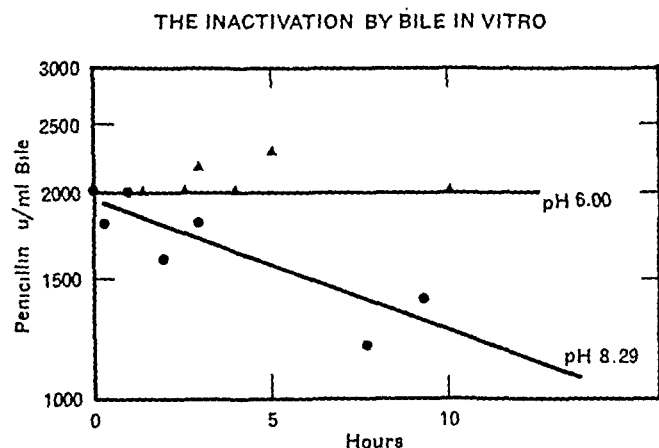


FIG. 4. THE LINES SHOW THE INACTIVATION, AT THE GIVEN pH VALUES AS CALCULATED FROM THE DATA OF BRODERSEN (4), IN GOOD AGREEMENT WITH THE EXPERIMENTAL POINTS

The two samples of bile were obtained from the gall bladder (pH 6.00) and from a common duct fistula (pH 8.29).

The pH values were determined electrometrically by comparison with 0.01 m. HCl + 0.5 m. NaCl (pH 2.00). The hydrogen ion concentrations were found to be independent of temperature in the range of 20 to 40° C.

so, the initial drop in serum concentration might be due to excretion of bile into the biliary system to the point of distention. However, it appeared still possible that destruction may have taken place in the liver, but that liver function was impaired by the biliary obstruction.

In another nephrectomized dog, the common bile duct was cannulated and the secreted bile collected hourly after an intravenous injection of penicillin. Very high concentrations of penicillin were found in the bile samples. The total amount recovered in the bile was 80 per cent of the injected penicillin. It could be concluded that, at least in this dog, the main part of the extra-renal elimination took place as excretion in the bile.

Inactivation by Bile In Vitro

A sample of dog's bile (pH 8.29), obtained from a fistula, was mixed with penicillin and incubated at 37° C. A rather slow inactivation was found to take place. Another sample drawn from the gall bladder (pH 6.00) did not inactivate penicillin at a demonstrable rate. This suggested that the inactivation was due to the alkalinity of the fistula bile. The rate of inactivation to be expected in this case was calculated (4) and found to be consistent with the experimental values

(Figure 4). The rate of inactivation in the bile can thus be calculated at known values of temperature and hydrogen ion concentration on the basis of the known destruction rate by alkali.

At the highest pH values found in the bile (8.65), the rate of inactivation found in the dogs with ligated bile ducts (Figure 3) could readily be accounted for as taking place in the bile standing in the intra-hepatic ducts.

The Relationship of Penicillin Concentrations in Serum and Bile

In Figure 5 are plotted the concentrations of penicillin in cannulated bile and in blood serum taken simultaneously from a nephrectomized dog. There was a constant ratio between the two concentrations, the bile containing 130 times as much penicillin per ml. as the serum at serum concentrations ranging from 100 to 5 units/ml. This ratio was found to vary considerably. In seven experiments carried out in two dogs, values ranging from 10 to 330 were obtained. From the figures in Table III it is seen that the high ratios generally were found at high pH values in the bile and the low ratios at the low pH values.

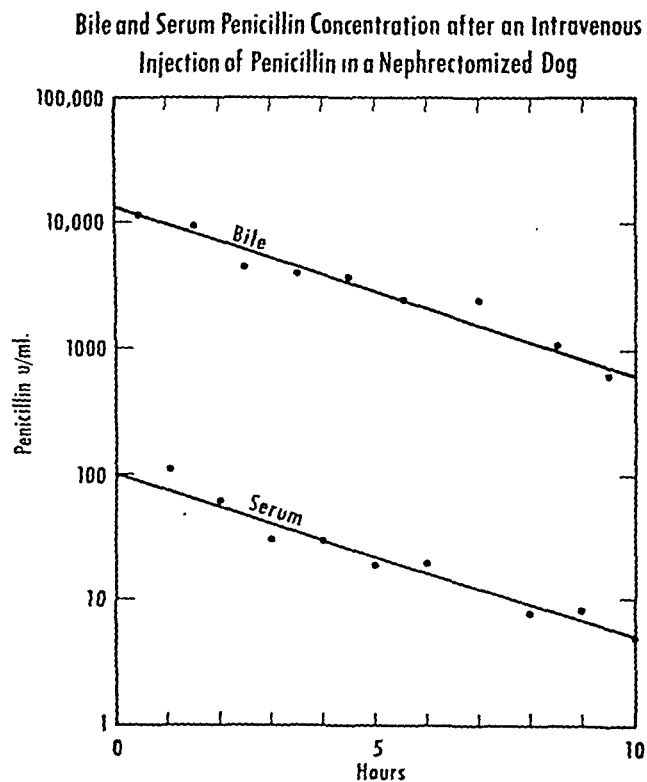


FIG. 5. THE BILE IS SEEN TO CONTAIN ABOUT 130 TIMES AS MUCH PENICILLIN PER MILLILITER AS THE SERUM, INDEPENDENT OF THE SERUM CONCENTRATION

TABLE III

The correlation between bile pH and the bile penicillin-serum penicillin ratio

pH of bile	$\frac{\text{Penicillin concentration of bile}}{\text{Penicillin concentration of serum}}$
8.65	330
8.50	130
8.48	270
8.29	10
8.20	16
8.04	11
7.93	11

Storage of Penicillin in the Gall Bladder

In a normal dog, a cannula was placed in the gall bladder and the common bile duct ligated in order to close the outlet from the gall bladder. The bile was drawn from the gall bladder, mixed with 100,000 units of penicillin and reinjected. Every hour the volume of bile in the gall bladder was determined and a small sample taken out for penicillin assay. The total amount of penicillin found in the gall bladder is shown in Figure 6. The pH values were determined at the same time and from these the spontaneous inactivation calculated as shown by the curve. The difference between this curve and the points demonstrates

RESORPTION AND INACTIVATION OF PENICILLIN IN THE GALL BLADDER

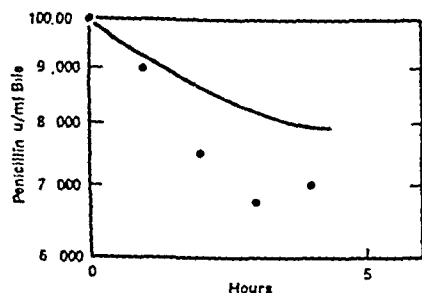


FIG. 6. THE CURVE INDICATES THE CALCULATED INACTIVATION

The experimental points are seen to deviate relatively little from this curve, indicating that only a slow resorption of penicillin takes place from the gall bladder.

the resorption from the gall bladder. Within the four hours of the experiment the resorbed amount is seen to be rather small. That a certain resorption does take place was shown by the fact that penicillin was present in the urine of the dog.

SUMMARY AND CONCLUSIONS

Bilaterally nephrectomized dogs were found to eliminate penicillin at a considerable rate from their blood stream after an intravenous injection. This process took place even if the intestines were removed, whereas no inactivation could be demonstrated after complete evisceration or hepatectomy. A major part of the penicillin was found to be excreted in the bile. A certain inactivation took place in the bile, especially when it was strongly alkaline. The remaining portion went either to the gall bladder, where only a slow resorption took place, or to the intestine, where it was partially resorbed and partially inactivated.

Caution must be exercised in drawing generalized conclusions from these results. Since the nephrectomized dogs do not eat, it seems possible that normal dogs might inactivate certain amounts of penicillin in their intestine because of a greater excretion of digestive juices.

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STUDIES ON HYPERSENSITIVITY TO PENICILLIN. I. INCIDENCE OF REACTIONS IN 1303 PATIENTS^{1, 2}

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Although penicillin is a relatively safe drug, many reactions, including three deaths (1-3), have been reported following its administration. Most of these reactions appear to be allergic in origin, even though the nature of the antigen has usually not been determined. During the years that penicillin was being purified many reactions were considered to be caused by impurities, particularly when the reaction was specific for a particular lot. When slowly absorbable preparations became available it seemed important to determine whether administration in this form increased the incidence of reactions. We have undertaken a study to determine the reaction rate to crystalline penicillin in aqueous solutions and to slowly absorbable forms as seen in ordinary hospital practice as well as factors influencing the incidence of such reactions and the nature of the antigen involved. In the present paper we are reporting the incidence of reactions which we have found and some of the factors believed to be important in determining the reaction rate. Studies on skin tests with penicillin oil and beeswax will be reported elsewhere (4).

METHOD

The method of this study was to observe daily all patients being treated with a preparation containing penicillin. Before treatment was initiated the patients were questioned regarding previous courses of penicillin and previous allergic reactions. A physical examination was then performed and the type of preparation, the dose, and the lot number of the penicillin employed were recorded. At least once a day each patient was examined and questioned closely regarding signs and symptoms of allergic reactions and of reactions at the site of injection. At

the start of the study it was anticipated that most of the patients would be given a second course of penicillin after an interval of several days in order to bring out latent sensitivities. It soon became apparent that this procedure produced very few allergic reactions so that the method was abandoned. As a result, in order to demonstrate statistically significant differences between the various preparations of penicillin we were forced to rely mainly on the observation of a large number of patients receiving each preparation.

The diagnosis of allergic reactions, where no confirmatory data were available (such as a positive reaction to a skin test or to a second course), was made on clinical grounds by the appearance of the reaction when penicillin was being administered and its disappearance shortly after the antibiotic was discontinued and by the fact that no other allergen could be indicted. Patients who developed reactions typical of serum sickness shortly after penicillin administration were considered penicillin reactors if no other reasons were found for such a reaction.

RESULTS

A total of 1303 patients who were under treatment for various infections were followed in this manner. As shown in Table I, 598 received aqueous crystalline penicillin, 557 were given crystalline penicillin in oil and beeswax, and 148 received procaine penicillin in oil. The incidence of allergic reactions in these groups was 1.2, 2.7, and 1.4 per cent respectively. The rates were significantly higher for the oil and wax group than for the aqueous group. There were not enough patients who received procaine penicillin to make the difference between this and the other groups statistically significant. An analysis of the effect of the size of the dose used on the incidence of reactions revealed that in the oil and beeswax and in the procaine penicillin groups, where maximum dose employed was only four times the minimum dose, no difference in the reaction rate with moderate and low doses was found. In the aqueous penicillin group, however, many of the doses used were ten or more times the usual doses employed. Thus, if the patients receiving 500,000

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TABLE I
Incidence of allergic reactions following administration of various penicillin preparations

Preparation	Dose	Duration						All cases	
		0-5 days		6-10 days		Over 10 days			
		No. of patients	No. reactions	No. of patients	No. reactions	No. of patients	No. reactions	No. of patients	No. reactions
Crystalline penicillin in oil and beeswax	Low, 300,000 U daily	153	3	77	4	40	2	270	9 (1)
	Moderate, over 300,000 U daily thru 600,000 U B.I.D.	145	3	94	1	48	2	287	6 (2)
Crystalline penicillin in aqueous solution	Low, 50,000 U q3h or less	176	0	184	2	123	1	483	3 (3)
	Moderate, over 50,000 U q3h to 500,000 U q2h	21	0	24	0	19	0	64	0 (4)
	High, over 500,000 U q2h	27	0	6	2	18	2	51	4 (5)
Procaine penicillin in oil	Low, 300,000 U daily	59	2	9	0	9	0	77	2
	Moderate, over 300,000 U daily to 600,000 U B.I.D.	39	0	22	0	10	0	71	0
Total		620	8	416	9	267	7	1303	24

All of the following are significant by the Chi Square Test. $1 + 2 > 3 + 4 + 5$
 $3 + 4 < 5$
 $1 > 3 + 4$

units or more every two hours are compared to those receiving less than this amount, reaction occurred in four among 51 patients (7.8 per cent) and in three among 547 patients (0.6 per cent) respectively. This difference is statistically sig-

TABLE II
Comparison of incidence of allergic reactions to crystalline penicillin and sulfamerazine at different doses

Preparation	Dose	Patients	Reactions	
			Patients	%
Crystalline penicillin	Less than 500,000 U every 2 hours	547	3	0.6
Do	500,000 U. or more every 2 hours	51	4	7.8
Sulfamerazine	Initial dose 6 gm., then 1 gm. every 4 hours	35	2	5.9
Do	Initial dose 2 mg., then 0.5 gm. every 4 hours	87	3	3.4
Do	Initial dose 1 gm., then 0.5 gm. every 8 hours	58	1	1.7

nificant when tested by the chi square test. Table II compares these rates with similar figures obtained by us for one of the sulfonamides (sulfamerazine). There is a trend in our figures which indicates that the incidence of reactions increased as the duration of therapy was prolonged although there are too few reactions to be certain of this point.

Two factors found to be of significance in the

TABLE III
Relationship of history of previous allergy to reactions to penicillin

Group	History of allergy	No. history of allergy
Non-reactors in controlled group	177	848
Reactors to penicillin in controlled group	7*	13*
Reactors in controlled group believed to be allergic to vehicle used	1	3
All reactors to penicillin	18	22
All reactors believed to be allergic to vehicle used	1	4

* 7/184 is significantly greater than 13/861 by Chi-Square Test.

TABLE IV
*Relationship of previous penicillin administration
to reactions to penicillin*

Group	History of receiving previous penicillin	
	Yes	No
Non-reactors in controlled group	21	376
Reactors to penicillin in controlled group	8*	12*
Reactors in controlled group believed to be allergic to vehicle used	2	2
All reactors to penicillin	18	22
All reactors believed to be allergic to vehicle used	3	2

* 8/29 is significantly greater than 12/388 by Chi² Test.

incidence of reactions to penicillin are a previous allergic history and the previous administration of penicillin. Tables III and IV illustrate these data. Reactions believed to be caused by penicillin itself and by the vehicles used (as determined

by second courses and/or skin tests) are listed separately. The patients in this, the controlled series, who showed reactions to penicillin are listed alone and are also included in the total group of reactors to penicillin. This total includes, in addition, the penicillin reactors whom we observed from all other available sources. In the controlled group there is a significantly greater reaction rate among individuals with such a history, and such a history was obtained in 35 per cent of the penicillin reactors. Since some of the patients outside of the controlled series who were available to us for study following a penicillin reaction were obtained from a private practice in allergy, the overall group is weighted in favor of persons with a history of some previous manifestation of allergy.

The high incidence of reactors who had received previous penicillin is of interest (Table IV). In both the controlled series and the total group there was a large percentage of patients who had

TABLE V
Local reactions to various penicillin preparations

Preparation	Dose	Duration of treatment												All cases			
		0-5 days				6-10 days				Over 10 days				No. of patients	Reactions		
		No. of patients	Mild	Mod.	Severe	No. of patients	Mild	Mod.	Severe	No. of patients	Mild	Mod.	Severe		Mild	Mod.	Sev.
Crystalline penicillin in oil and beeswax	Low, 300,000 U daily	118	11	4	2	51	8	5	2	16	5	3	3	185	24	12	7
	Moderate, over 300,000 U daily thru 600,000 U B.I.D.	85	25	6	3	28	10	6	2	19	11	4	4	132	46	16	9
Crystalline penicillin in aqueous solution	Low, 50,000 U q3h or less	141	10	1	0	140	7	0	0	116	13	2	1	397	30	3	1
	Moderate, more than 50,000 U q3h to 50,000 U q2h	16	0	1	0	12	2	0	0	2	0	0	0	30	2	1	0
	High, 500,000 U q2h and over	17	6	3	0	3	1	1	0	10	4	0	1	30	11	4	1
Procaine penicillin in oil	Low, 300,000 U daily	28	4	0	0	9	1	0	0	9	0	0	0	46	5	0	0
	Moderate, more than 300,000 U daily thru 600,000 U— B.I.D.	36	8	0	0	20	3	0	0	10	1	0	0	66	12	0	0
Total		441	64	15	5	263	32	12	4	182	34	9	9	886	130	36	18

previously received penicillin (40 and 45 per cent, respectively). In the controlled group the reaction rate among patients who had previously received penicillin was significantly higher than the rate among those to whom the penicillin was given for the first time (28 per cent versus 3 per cent).

Reactions in the controlled group were somewhat more frequent in males (12 reactions among 683 males) than in females (eight out of 600). The incidence of reactions in the white patients (eight in 389 patients) was greater than that for the Negroes (12 in 894 patients), but this is not statistically significant.

We had little success in demonstrating latent allergy by giving second courses routinely. Among 122 patients who received second courses of aqueous crystalline penicillin only one had a reaction, while 111 patients received a second course of crystalline penicillin in wax and oil and 28 a second course of procaine penicillin without exhibiting a reaction. Almost all of the intervals allowed between the first and second courses were under ten days, and the majority were three, four, or five days. The one reaction occurred on the third day of the second course, ten days following the first course.

Eight hundred and eighty-six patients were studied by daily observation for the occurrence of local irritative reactions, which were graded as mild, moderate and severe depending upon the degree of pain, tenderness, induration, edema, and redness present. It was found (Table V) that penicillin in oil and wax caused significantly more irritative reactions than aqueous crystalline penicillin (36 per cent compared with 12 per cent). Procaine penicillin in oil although it caused more mild local reactions than aqueous penicillin produced no moderate or severe reactions at all, and therefore in the doses used appeared to be the least irritating of the three preparations. In the case of penicillin in oil and wax and aqueous penicillin, the dose and duration of treatment appeared to be directly related to the incidence and severity of the local reactions.

DISCUSSION

The incidence of penicillin reactions has been reduced by the use of crystalline penicillin. Keefer and his colleagues (5) using crude penicil-

lin reported 69 reactions among 500 patients although it is uncertain how many of these were allergic in nature. Lyons (6) reported urticaria in 5.7 per cent of 209 patients receiving crude penicillin. Duemling (7) reported a 10 per cent reaction rate including Herxheimer reactions in 17,879 patients treated for 65 different conditions. The patients were treated over the years during which penicillin was being purified and thus penicillin of varying degrees of purity was used in these patients. Thomas and co-workers (8) reported 2.5 per cent of 10,000 syphilitics developed urticaria following therapy. Peck and co-workers (9) observed 32 reactions among 130 patients who received crystalline penicillin. The overall incidence of reactions in our series of 1.2 per cent for aqueous crystalline penicillin patients, 2.7 per cent for crystalline penicillin in oil and beeswax and 1.4 per cent for procaine penicillin in oil are compatible with most of these figures. Since some of our patients were discharged a few days after penicillin was discontinued and since we know that certain delayed penicillin reactions may occur after a longer interval than this even when the aqueous preparation is used, the reported rates may be somewhat low.

It is of interest that there are significantly more reactions when the penicillin is administered in oil and beeswax. Even though three of these reactions may have been caused by the vehicle (10), this does not account for all of the difference. The dose used is evidently not the key to the situation since the penicillin in oil and wax doses did not exceed the moderate range and when the reactions in this group are compared with those occurring in the aqueous penicillin patients who received comparable doses, the greater tendency of the oil and wax preparations to cause reactions is even more pronounced. Moreover, moderate doses of penicillin in oil and wax gave no higher reaction rates than the low doses (300,000 units per day). Two factors which may be of importance are that the penicillin is held in contact with the tissues in one site over a prolonged period of time, and in the same area as evidenced by the local irritation there are probably many damaged proteins which may conjugate with the penicillin and render it antigenic. Observation of a larger group of patients under treatment with procaine penicillin may indicate whether the prolonged

contact or the irritative component is more important. That more reactions of the delayed type occur after the use of penicillin in oil and wax than after the use of aqueous penicillin may indicate that the prolonged contact locally or systemically is important. Some of these reactions, however, are probably caused by a metabolic product of penicillin particularly since they do follow the use of aqueous penicillin. Inasmuch as the skin is an important organ for causing penicillin sensitization it is possible that the minute quantities of penicillin in oil and wax escaping into the cutaneous tissue are important. Small amounts of wax and oil will remain in the skin for a prolonged period when injected intracutaneously (4) and may hold penicillin there in contact with the tissue for some time.

The fact that the employment of very high doses of aqueous penicillin results in a high reaction rate may depend on the fact that these large amounts produce considerable local irritation, even though for only a relatively brief period, with the result that more proteins are available to conjugate with the large amount of penicillin present at the site. On the other hand the concentration of penicillin or a metabolic product of it in the distant organs may be more important. In this connection the fact that when high doses of penicillin are given the reaction rate approximates that obtained with a sulfonamide indicates that penicillin is rather allergenic in large doses and that the difference between it and the sulfonamides may be mainly a question of dosage.

Our data indicate that an underlying allergic diathesis may be important in the development of penicillin allergy. We have examined the histories of 138 patients reported in the literature. Among the cases in which a history of allergy is mentioned, the history was positive in 18 per cent.

Among the 138 histories examined, 63 patients had previously received penicillin therapy, locally or systemically, at least once and 16 patients had received none. No data on previous penicillin administration were recorded for the 59 remaining patients. If the latter patients are omitted from the calculation, about 80 per cent of the reactors had previous contact with penicillin. Our figures are in agreement with a high incidence of previous exposure.

In this connection our failure to demonstrate "latent" allergy by administering a second course of penicillin is of interest. It is possible that the interval allowed in many of the patients was too short, since we have observed delayed reactions after the discontinuance of penicillin with incubation periods longer than the time which we allowed between courses in most patients. Furthermore, the intervals in the patients who reacted to penicillin treatment were much longer both in our cases and in those reported, usually a matter of months rather than a few days.

Peck and co-workers (9) have reported that the incidence of reactions is much higher among males than among females. Although the rate was somewhat higher among our male patients than among our female patients, the difference was not statistically significant. The reaction rate in Negroes may be lower than that for white patients. This problem deserves further study.

The fact that the incidence of allergic reactions and local irritative reactions seem to parallel one another may indicate a cause and effect relationship as discussed above. This is not necessarily so, however, since several mechanisms are available to explain the production of each phenomenon. Certainly some patients with severe local reactions do not have allergic reactions whereas others with no demonstrable local reaction may have severe allergies so that even if the two phenomena are related other determinants may be operative.

SUMMARY

1. The incidence of allergic reactions was studied in 1303 patients. Among 598 patients receiving aqueous crystalline penicillin the rate was 1.2 per cent, among 557 patients receiving crystalline penicillin in oil and beeswax it was 2.7 per cent, and among 148 patients receiving procaine penicillin in oil it was 1.4 per cent. In patients receiving very large doses of the aqueous preparation the reaction rate was high (7.8 per cent). Reactions were more frequent when a product was administered for a longer period of time.

2. Reactions were more frequent among patients who gave a history of previous allergy or who had previous known contact with penicillin. The effect of sex and race on the incidence of reactions was not significant in this group of patients.

3. Local irritative reactions were more frequent and more severe with penicillin in oil and beeswax than with aqueous crystalline penicillin and procaine penicillin in oil. Higher doses and/or more prolonged therapy were likely to be accompanied by a higher incidence of irritative reactions.

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THE RECOVERY OF BACTERIA FROM THE TOXIC EFFECTS OF PENICILLIN¹

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There is considerable evidence that the therapeutic action of penicillin is determined primarily by the total length of time for which it remains at concentrations effective against the particular organism. It is, however, equally clear that the exposure to penicillin need not be continuous. Injections of aqueous penicillin may be given so infrequently that for an appreciable period of time the serum concentrations of the drug are below the limits effective against the particular organism, and yet such widely-spaced injections may be fully effective therapeutically. This raises the question as to why the bacteria fail to remultiply during the time that the serum concentrations are less than that necessary to kill the organisms *in vitro*. Several of the possible explanations are apparently not valid. We have not found the bacteria we have studied to be appreciably more sensitive to penicillin *in vivo* than they are *in vitro*. The concentrations necessary to effect cure are of the same order of magnitude as those required to kill the bacteria in the test tube. Similarly, we have not found that penicillin, after its intramuscular injection in aqueous solution, persists at effective levels in the tissues for a significantly longer period than in the blood.

This paper will describe some experimental data which may throw some light on this problem. The clue was the observation by Parker and Marsh (1) that after staphylococci had been exposed to penicillin, they did not immediately remultiply on the removal of the drug. Instead, there followed a period of recovery during which they remained essentially constant in number, and during which they were presumably recovering from the toxic effects of the drug. We have found this phenomenon to be observed with every bacterial species so far studied, whether with group A, group B or

group C β -hemolytic streptococci, α -hemolytic streptococci, type I or type III pneumococci, *Staphylococcus aureus*, *Staphylococcus albus*, or *Streptococcus fecalis*. After the bacteria have been exposed to penicillin, on the removal of the drug there follows a variable recovery period during which their numbers remain constant for a period of two, four, six, or even eight to 10 hours. Only after this period of recovery do the organisms begin to multiply at their normal rate (Figure 1). In some instances the toxic effect on the bacteria was manifested by a reduced rate of multiplication on the removal of penicillin, rather than an absolute bacteriostasis (Figure 2).

The rate at which this toxic effect is initiated, and the duration of the following recovery period, vary with individual bacterial species, and are affected also by two additional factors. One is the concentration of the drug to which the bacteria had been exposed (Figure 3). The toxic effect is initiated only by concentrations eventually bactericidal to the organism. The rate at which the toxic effect develops varies with the concentration of penicillin, and reaches a maximum at the concentration most rapidly bactericidal for the particular organism. Beyond that maximum, even a 10,000-fold increase in the concentration of penicillin does not further accelerate the rate at which the toxic effect is initiated.

The duration of the recovery period is affected also by the time for which the bacteria had been exposed to penicillin. With some strains (Figure 4) the toxic effect reaches its maximum within one to two hours, and even 24-hour exposure to penicillin does not significantly prolong the duration of the following recovery period. With other strains, however, the duration of the recovery period increases directly and continuously with the duration of the preceding exposure to penicillin (Figure 5).

It is apparent that a recovery period during which the organisms remain static in number provides a valuable margin of safety in the thera-

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

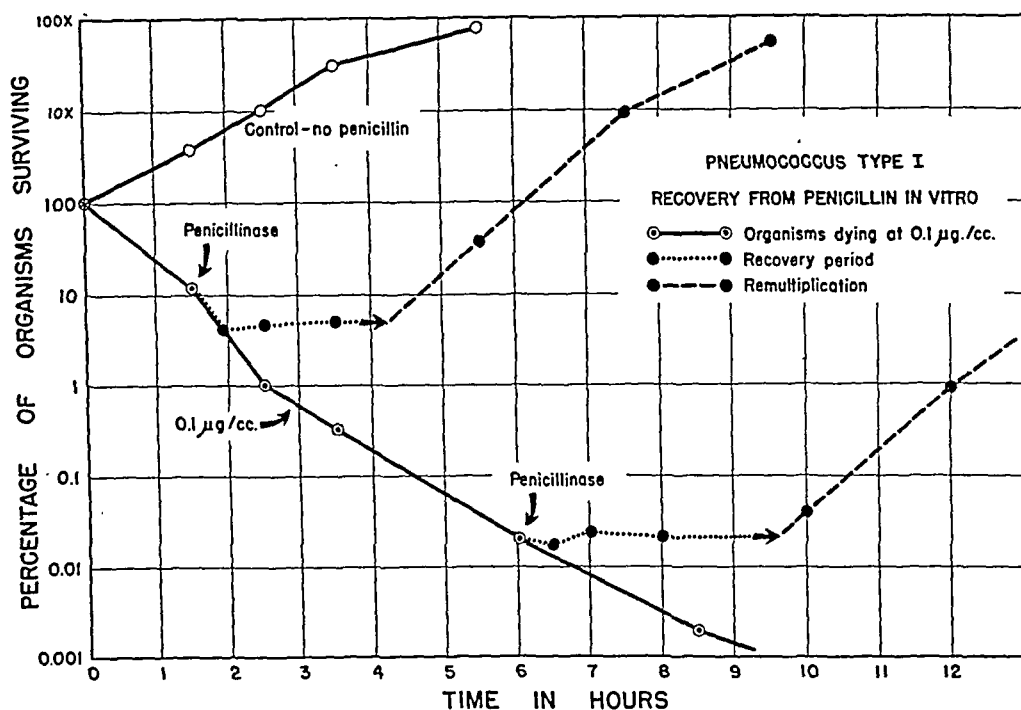


FIG. 1

peutic use of the drug, a period during which the organisms will not multiply even in the absence of penicillin. The therapeutic importance of this phenomenon, however, goes beyond the mere failure of the bacteria to multiply. We have found

that during this recovery period, bacteria previously exposed to penicillin are extraordinarily susceptible to the defense mechanisms of the body. Even highly virulent organisms against which the body ordinarily has so little defense that one or

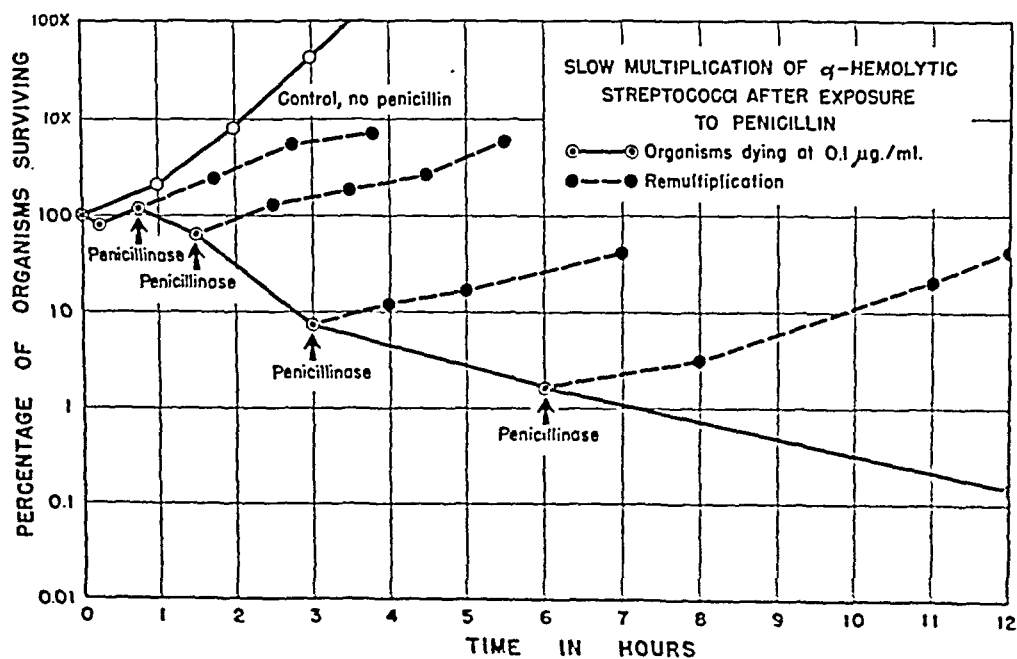


FIG. 2

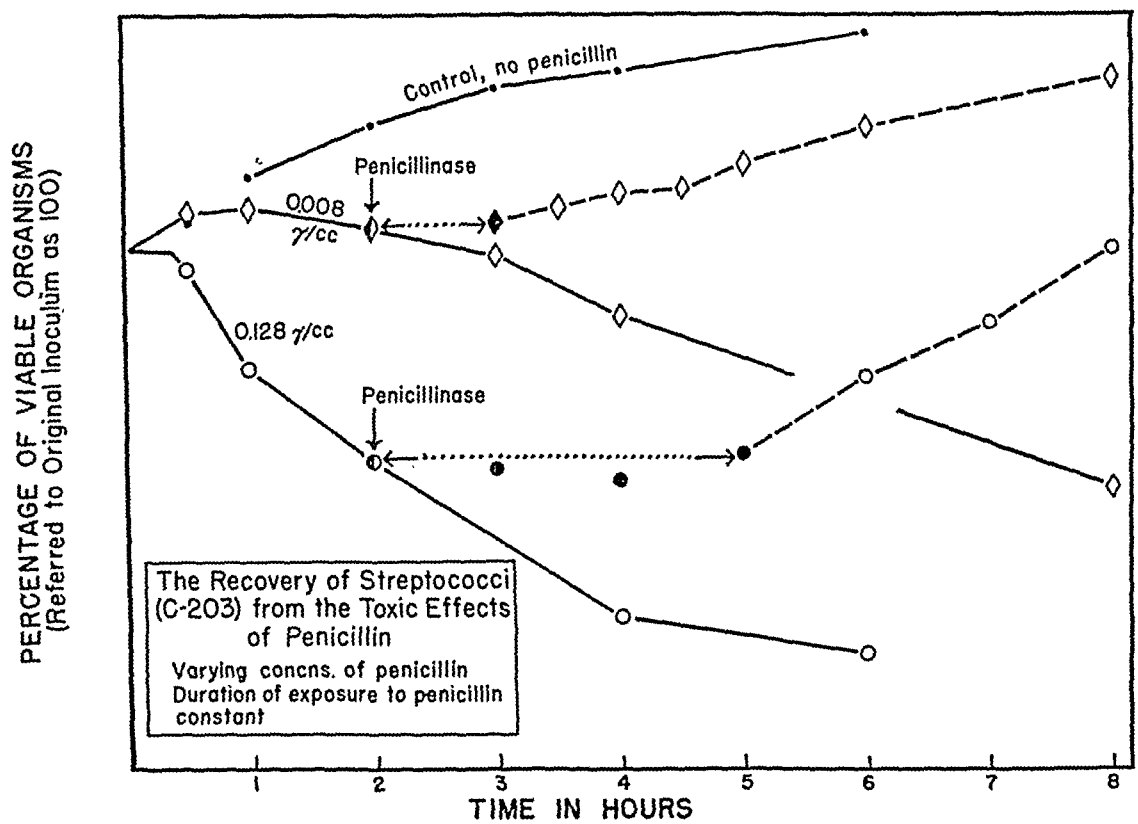


FIG. 3

two bacteria suffice to initiate a fatal infection become relatively avirulent after exposure to penicillin. In order to initiate a fatal infection, one must inject, not one, but 100, 1,000, or in several ex-

periments even 5,000 of these recovering but viable bacteria. As the bacteria recover from the toxic effects of penicillin, their virulence slowly returns, reaching its normal level at the time

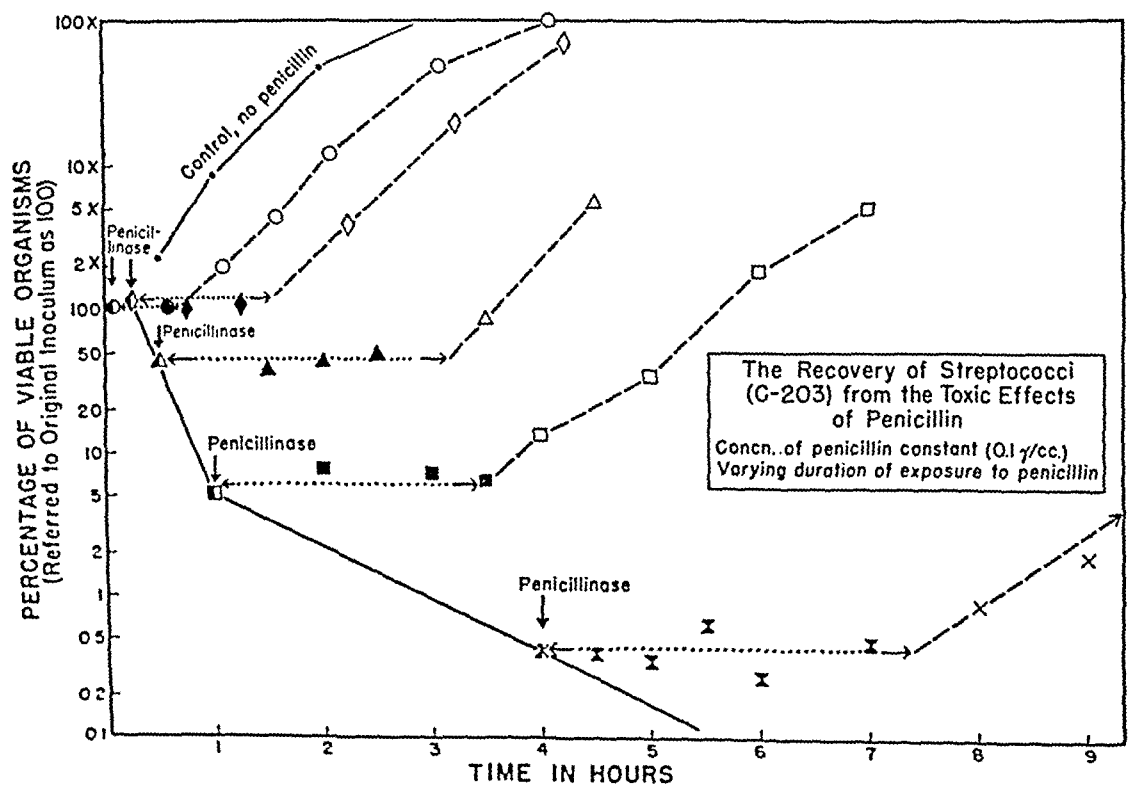


FIG. 4

RECOVERY FROM PENICILLIN

(GROUP B) β -HEMOLYTIC STREPTOCOCCI (256 $\mu\text{g/ml}$)

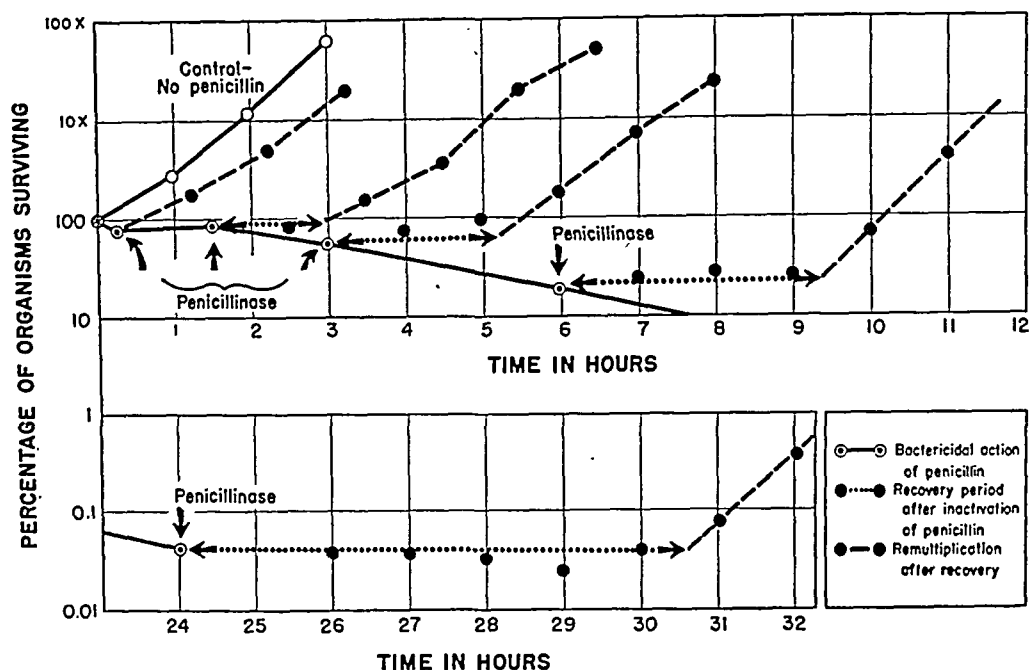


FIG. 5

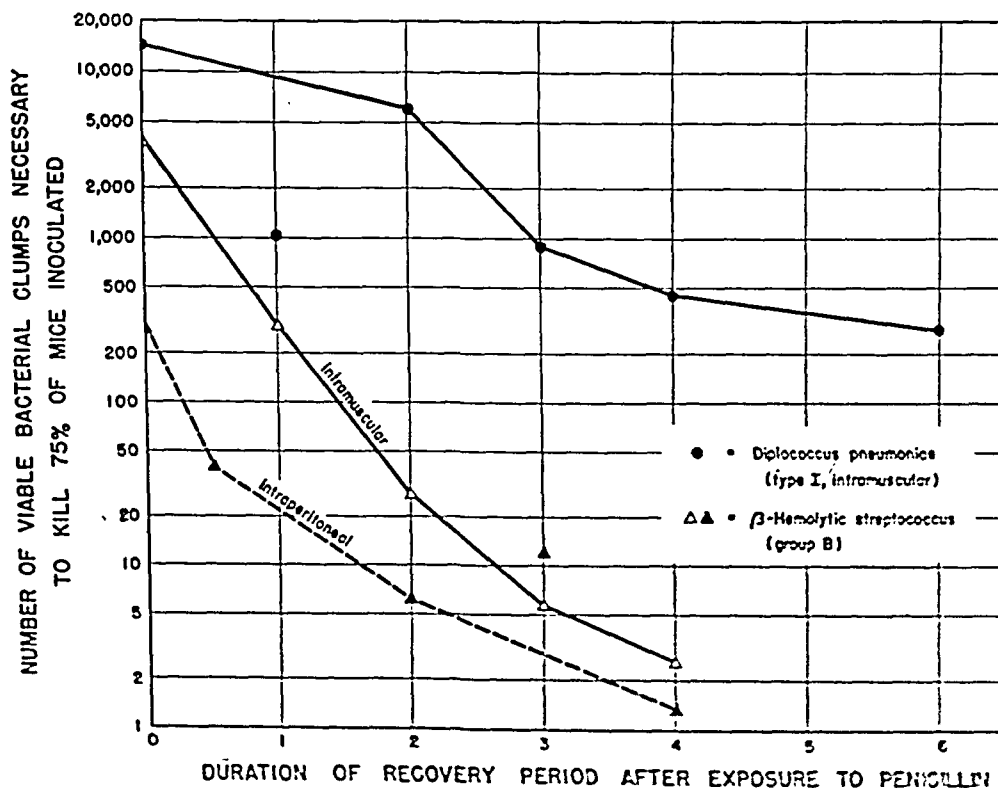


FIG. 6

that the bacteria resume multiplication in the test tube (Figure 6). This decreased virulence of bacteria which have been acted on by penicillin can only mean that the organisms have become susceptible to the defense mechanisms of the host. One must inoculate as many as 100 or 1,000 bacteria in order to have at least one organism surviving by the time the bacteria have recovered sufficiently to resume multiplication and regain their normal virulence.

The therapeutic implications are two-fold. In the first place, bacteria should be, and are, killed by penicillin even more rapidly *in vivo* than they are *in vitro*. The bactericidal action in the infected animal is the sum of the direct effect of penicillin itself, plus the bactericidal action of the host mechanisms on organisms which are being acted on by the drug and thereby rendered susceptible. This has been found to be the case in a number of acute experimental infections of rabbits and mice. The relative quantitative importance of these two mechanisms is under present study. In the second place, the therapeutic action of penicillin persists well beyond the time for

which the drug remains at effective levels. For a varying period of time after penicillin has fallen below bactericidal concentrations, the damaged but viable bacteria continue to die under the impact of the host defense mechanisms. There is reason to believe that the number of bacteria killed during this penicillin-free period is usually less than those killed by the drug and the host during the period of penicillin action. The recovery period is nevertheless of major importance in that it provides a necessary and desirable margin of safety in the therapeutic use of penicillin. Even though the total length of time for which penicillin remains at effective levels may be the factor primarily determining its therapeutic efficacy, those levels need not be continuously maintained.

Studies are in progress to determine whether humoral or cellular host mechanisms are concerned in the death of bacteria which have been damaged by penicillin.

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AMPEROMETRIC MICRO-TITRATION OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN¹

By JOHN B. CONN AND SARA L. NORMAN

(From the Research Laboratories of Merck & Co., Inc., Rahway, New Jersey)

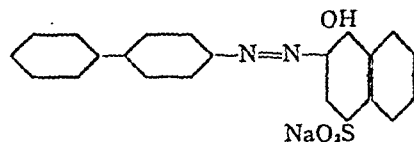
With the introduction of dihydrostreptomycin into general use as an antibiotic, the development of a chemical assay procedure becomes desirable. Whereas adequate chemical methods exist for streptomycin, based on the characteristic maltol reaction (1, 2), dihydrostreptomycin does not respond similarly, and other techniques must be sought.

One property which is shared by both streptomycin and dihydrostreptomycin is the formation, under certain conditions, of sparingly soluble to insoluble salts with various anionic dyes, some of which are crystalline (3, 4). Since these dyes are polarographically reducible, it seemed possible that the precipitation reaction might be utilized in an amperometric titration procedure, which would have the advantage of being independent of other assay methods. When appropriate investigation was launched, it soon became apparent that no simple reaction was being dealt with. While a wide variety of acid azo dyes were found to give precipitates with dihydrostreptomycin salts in aqueous solution, the precipitates tended to be peptized by inorganic salts in concentration above a critical level or by excess dye, and further, would not separate at all unless the pH of the medium were held to a very narrow range. Inasmuch as polarographic methods require the presence of a supporting buffer electrolyte of concentration many times that of the component being measured, it was evident that dyes fulfilling the requirements would be rare.

Altogether, some seventy-odd dyes have been tested during the course of this work, of which 60 were commercial products, the remainder synthesized for the purpose. None of the commercial samples could be made to function in the desired manner; of the synthetics, those which showed promise were derived from diazotized 4-

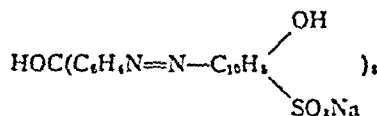
aminobiphenyl and diazotized pararosanine, coupled with 1-naphthol-4-sulfonic acid.

The first of these, having the structure



would precipitate dihydrostreptomycin in 0.1N borate buffers of pH 7.5–8.5, but it was at once apparent that the composition of the precipitate was not the expected BA_3 , but contained much less dye. Moreover, a slow but steady drift toward zero occurred in the diffusion current after each addition of dye, once the equivalence point had been passed. Titrations carried out rapidly gave sharp apparent equivalence points, but the results were too erratic to be of value. After fruitless attempts to surmount the difficulty, which was caused by ion exchange between the precipitate and excess dye in solution, the use of this dye was abandoned.

A priori, the solution to the problem of variable precipitate composition would be to select a dye which could give a dihydrostreptomycin salt of but one type; namely, one having three acidic functions. The representative most easily prepared by unequivocal method has the structure



Trisodium tris-(naphtholazophenyl) carbinol trisulfonate

produced by diazotization of pararosanine and coupling with 1-naphthol-4-sulfonic acid. The red dye is soluble in water up to 0.05M (5%) and precipitates dihydrostreptomycin in 0.01M triethanolamine citrate buffer, pH 2.8, without any troublesome drift effects. (The use of triethanolamine citrate as supporting electrolyte was dictated by the peptizing action of the common inorganic cations.) The composition of the dihy-

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11–12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

drostreptomycin dye precipitate is not exactly stoichiometric, but is reproducible under the conditions of titration.

METHOD

Preparation of materials

It is essential that pure intermediates be used in the preparation of the dye; otherwise mixtures of variable behavior result. Neither commercial fuchsine, which consists of a mixture of pararosaniline and its methyl and dimethyl homologues, nor technical grades of 1-naphthol-4-sulfonic acid can be used. Eastman Kodak best grade pararosaniline base is suitable, while a pure grade of 1-naphthol-4-sulfonic acid is easily prepared from Eastman Kodak best grade 1-naphthylamine-4-sulfonic acid via the Bucherer reaction (5).

Suspend 15.3 g. (0.05 mole) finely pulverized pararosaniline base in 500 ml. water, cool to 5°, add 27 ml. conc. hydrochloric acid, and diazotize by dropwise addition of 10.4 g. (0.15 mole) sodium nitrite in 100 ml. water over the period of one hour, with continuous stirring. At the end of the process, the solution should be orange and no particles of unreacted pararosaniline chloride should be present. The diazonium salt solution is poured with stirring into a solution of 37 g. (slight excess over 0.15 mole) pure 1-naphthol-4-sulfonic acid in 500 ml. water containing 14 g. sodium hydroxide, chilled to 5°. The dark red solution is stirred for one hour at 5°, filtered, and treated with 10 g. ammonium chloride (to lower the pH), after which it is allowed to stand at room temperature overnight. The dye solution is salted out with sodium chloride and centrifuged; the precipitate is suspended in sufficient water to render the mixture fluid, poured into a cellophane sac, and dialyzed against running water for 36-48 hours to remove diffusible electrolytes (it has not been found possible to date to crystallize the dye). The dialyzed solution is concentrated to dryness on the steam bath, and the vitreous green residue further dried at 100°. Yield, 50.4 g. (93%). M.W., 1079.

For preparation of the titrating solution, 1.08 g. (0.001 mole) is dissolved in 100 ml. water by warming; the solution is allowed to stand for two days, and then filtered through a layer of Celite to remove any insoluble particles.

Supporting buffer: Dissolve 29.8 g. (0.2 mole) commercial triethanolamine and 21.0 g. (0.1 mole) citric acid in 1 liter water and mix 400 ml. of this solution with 600 ml. 0.1N HCl. This stock solution is diluted 1:10 with distilled water for use; the pH should be 2.8.

Procedure

A Leeds and Northrup Electro-chemograph was used in conjunction with a Lingane-Laitinen cell (6), the equipment being housed in a room thermostated at 25°. The burette was a 1 ml. mercury-expulsion type of the Gilment design.

The potentiometer is set at -0.8 v. vs. S.C.E., and recorder range at 10 micro-amperes. The cell is charged

with 5-10 ml. buffer, and a sample of dihydrostreptomycin salt equivalent to 1-3 mg. base is introduced either in a small platinum boat or as a measured amount of solution. After nitrogen is passed through the solution for five minutes, the gas flow is switched to overpass and a chart tracing obtained after establishment of equilibrium, the chart being allowed to run until a straight line is drawn, stopping for convenience on a chart division. Nitrogen is once more passed through the solution, and 0.05 ml. of dye is introduced, after which a second chart tracing is obtained. This cycle of operations is repeated until the equivalence point has been passed and four or five current steps of about equal magnitude are obtained. The chart is read off, the current values corrected for volume change and the results plotted.

Standard preparations of streptomycin and dihydrostreptomycin salts are employed for calibration purposes. If streptomycin hydrochloride-calcium chloride double salt is used, the calcium chloride is best removed, so as to avoid any disturbing effect due to its presence. This is easily accomplished by stirring the solution of the sample with silver carbonate until the pH rises above 8; the solid is removed by centrifugation, washed, and the combined solution phases brought to pH 5 with dilute acid.

RESULTS AND DISCUSSION

Table I lists data which have been collected on the titration of various dihydrostreptomycin salts.

In view of the usual difficulties of solvation and hygroscopicity which are encountered in dealing with these salts, the agreement is considered good, particularly since the amperometric titration depends on a property of the ion as a whole rather than any functional part.

TABLE I
Titration of dihydrostreptomycin salts

Salt	Ampero- metric assay	Other assays
Sulfate	734 743	
Chloride	808 817	
Sulfate	760 765	
Sulfate	782 790	765 (Base titration)
Sulfate	737 749	763 (Iodimetric)
Streptomycin Hydro- chloride-Calcium Chloride	748 733	740 (Maltol)

TABLE II
Stability of dihydrostreptomycin
in normal sulfuric acid

Time (hours)	Temperature	Decomposition (%)
1	100°	100
23	25°	25
95	25°	77

In practice it is found that separate standardizations are required for streptomycin and dihydrostreptomycin salts, since the molecular compositions of the salts are neither exactly stoichiometric nor identical. This effect is not unexpected, in view of the colloidal nature of the anion.

Since, as has been previously pointed out, inorganic salts exert a strongly peptizing action on streptomycin and dihydrostreptomycin tris-(naphtholazophenyl) carbinol trisulfonate, applications of the amperometric assay are limited to those in which such salts are either absent or removable. For example, in case it is desired to use the method for a study of the stability of dihydrostreptomycin in aqueous solution at different pH's and temperatures, the employment of sulfuric acid and barium hydroxide as acid and base permits removal of extraneous ions. The data in Table II on the acid stability of dihydrostreptomycin were obtained in this manner.

SUMMARY

Streptomycin and dihydrostreptomycin form water-insoluble salts with various anionic dyes; this property may be utilized for a precipitometric titration assay, provided that the characteristic tendency of the precipitate to be peptized by excess of indifferent salts can be overcome. In addition,

unless the dye anion is trivalent, precipitates of variable composition are likely to be produced.

The dye formed by coupling diazotized parosaniline with 1-naphthol-4-sulfonic acid precipitates streptomycin and dihydrostreptomycin quantitatively in 0.02M triethanolamine citrate buffer, pH 2.8. The polarograph is used in an amperometric titration procedure; an amount of streptomycin or dihydrostreptomycin salt corresponding to 1-3 mg. of base dissolved in 5-10 ml. buffer is titrated with 0.01M solution of dye, using a 1 ml. micro-burette; the polarograph is set at -0.8 v. (vs. S.C.E.) and 10 micro-ampere range.

The titration is reproducible to better than $\pm 1\%$. Using pure streptomycin and dihydrostreptomycin salts as standard, the method gives results in good agreement with other assay procedures.

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A NEW LINEAR DIFFUSION METHOD FOR THE MICROBIOLOGICAL ASSAY OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN¹

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(From the Lilly Research Laboratories, Indianapolis, Ind.)

A new antibiotic assay method has been developed which involves a radically different diffusion system from that employed in plate methods. In this new method diffusion occurs from a solution of the antibiotic in a test tube into a column of agar contained in a glass capillary.

This new method promises improved accuracy because the linear diffusion system has important advantages over the diffusion system presented in the plate methods. Secondly, this method provides greater efficiency in handling test equipment than is possible for other methods. Capillaries can be filled, read, and cleaned by semiautomatic procedures. Test tubes and other essential equipment are efficiently cleaned in a special steam cabinet. Finally, the method affords comparative economy in personnel and materials over other methods.

The method is inherently capable of extension to testing of other antibiotic or germicidal materials, but has been worked out in detail and applied only for the testing of streptomycin and dihydrostreptomycin. The obvious extension to testing of penicillin is being studied.

The test depends upon the formation of characteristic zones in the seeded agar column in the capillary. Figure 1 shows a section of a capillary in which such zones have formed.

A sharp initial boundary for diffusion of antibiotic from solution into the agar column is provided by the molded end of the agar column. A region of inhibition of growth occurs at this end of the agar column. The length of this region depends on concentration of antibiotic in the test solution. This region is bounded by a sharp dense zone of aerobic growth of the test organisms which allows the length of this region to be accurately measured. This sharp zone of aerobic growth results from the fact that oxygen diffuses unused

through the region of inhibition and becomes available to the first uninhibited organisms encountered. Since this growth uses all the oxygen available by diffusion inward, the growth of organisms beyond this zone is limited by the oxygen originally dissolved in the agar. Accordingly, growth in this region is very light. A pellicle of dense aerobic growth is always seen at the top surface of the agar column.

Figure 2 shows the manner in which the length of the inhibition region depends upon the concentration of dihydrostreptomycin in test solutions. Such a standard curve is prepared each day from known dilutions of a standard solution. The results on unknown solutions are referred to this curve for evaluation.

METHOD

A complete description of the required equipment and the details of the method is being published elsewhere. Only a brief description of the operations involved in the use of this method can be given here.

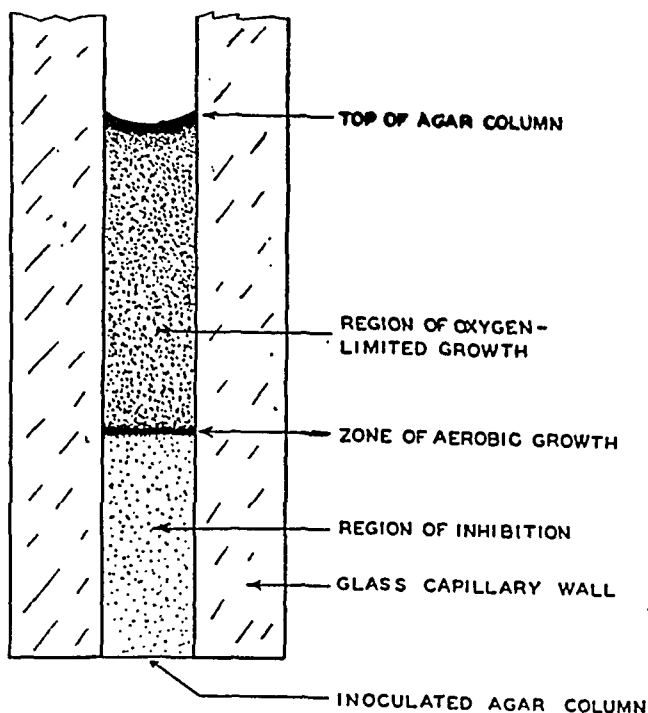


FIG. 1. SCHEMATIC DRAWING SHOWING THE REGIONS OF GROWTH IN THE AGAR COLUMN

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

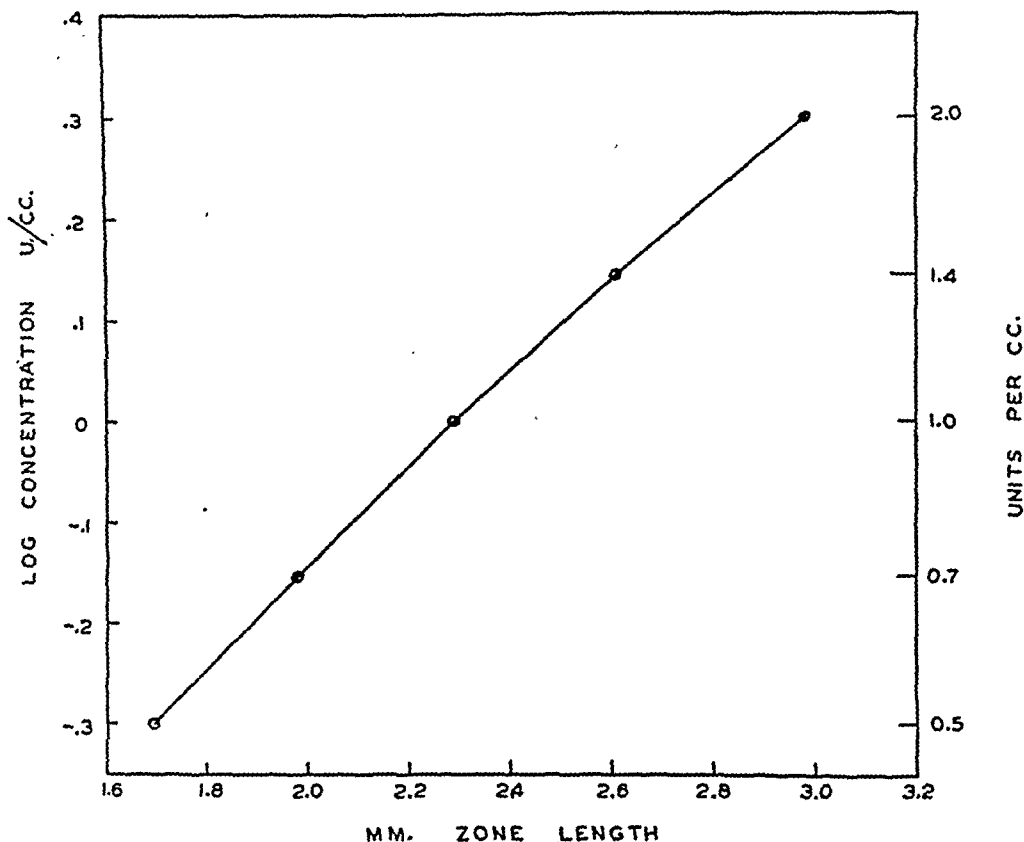


FIG. 2. A TYPICAL CURVE SHOWING THE DEPENDENCE OF ZONE LENGTH ON CONCENTRATION OF ANTIBIOTIC

The capillaries employed are constructed of pyrex capillary tubing of approximately 1.5 mm. bore and approximately 7 mm. outside diameter. They are cut to approximately $3\frac{1}{2}$ inches. One end is ground flat while the other is only edge ground.

Clean dry capillaries are partially filled by placing them in an automatic filling device containing plain 4% melted agar seeded with spores of *Bacillus subtilis*. This device fills 800 capillaries simultaneously. After the capillaries are filled, the entire assembly is cooled to allow solidification of the agar. This operation provides a flat molded end on the agar column in each capillary.

After cooling, the filled capillaries are removed from the filling device and placed two each in test tubes containing roughly 2 ml. of test solution. The test solutions have been made up by diluting standard and unknown antibiotic solutions with nutrient broth. The concentrations of standard test solutions are from 0.5 to 2.0 units per ml., while the unknowns are diluted to bring them to approximately 1 unit per ml. The test tubes are capped and incubated in racks in a constant temperature water bath at 32° C. overnight. After incubation the contents of a group of test tubes (usually five) containing a single test solution are dumped on a draining device. The capillaries are rinsed with water and then dried on an absorbent surface.

The capillaries are then transferred in a group to a special carriage provided on a modified Fisher-Lilly Antibiotic Zone Reader. The modification of the reader permits the length of the zone of inhibition to be read reproducibly to plus or minus 0.01 mm.

After the reading, the capillaries are stacked in a wire basket and cleaned simultaneously in the steam cleaning cabinet. Test tubes and bottles used for dilutions are cleaned in a similar manner.

The filling device formerly employed allowed simultaneous filling of 400 capillaries. Three units of test equipment are now being constructed, each designed to fill and handle 800 capillaries per day. Each unit anticipates the performance of 57 six-tube tests or 114 three-tube tests in addition to a complete standard curve.

RESULTS AND CONCLUSIONS

Deviations of test results on a 1 unit per ml. solution have been studied employing the disc-plate method and the capillary-linear diffusion method. Fifty Petri dishes were employed in each plate-disc experiment and 50 test tubes in each capillary experiment. The standard deviations for six plate-disc experiments were from

7% to 10% for individual zones, and those for seven capillary experiments were from 3% to 8% for individual capillaries. A similar experiment was performed to study the deviations of the broth-turbidimetric assay method, employing 150 tubes. The standard deviation of this method was found to be 3%. These data indicate that

when the two diffusion assay methods are compared on the basis of equivalent numbers of plates and pairs of capillaries, the linear diffusion capillary method is somewhat superior to the plate method in precision. In comparison to the broth-turbidimetric assay method the capillary method is less precise but more convenient.

QUANTITATIVE DETERMINATION OF DIHYDROSTREPTOMYCIN BY PERIODATE OXIDATION¹

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Dihydrostreptomycin is produced commercially by the catalytic hydrogenation of streptomycin. The reaction involves the reduction of the aldehyde group in the streptose moiety of the streptomycin molecule to the corresponding primary carbinol group as evidenced by the failure of dihydrostreptomycin to react with carbonyl reagents or with alkali to produce maltol. As there are no chemical methods available for the direct determination of dihydrostreptomycin, it was desirable to investigate the development of such a method. A satisfactory procedure has been found and is based upon the measurement of formaldehyde liberated by the periodate oxidation of dihydrostreptomycin.

Periodate oxidation is a general method of cleaving the linkage between two adjacent hydroxyl bearing carbon atoms. If one of the hydroxyl groups is a primary carbinol, formaldehyde will be a reaction product (1). In the elucidation of the structure of streptomycin, it has been proven there is no primary carbinol group which will give rise to formaldehyde on treatment with periodate (2, 3). However, in the case of dihydrostreptomycin there is a primary carbinol adjacent to an hydroxyl, and formaldehyde is liberated by periodate oxidation. This has been observed experimentally by Lemieux, DeWalt and Wolf from (4). These workers showed that dihydrostreptomycin when oxidized with 1.5 mole of periodate yielded 0.5 mole of formaldehyde while streptomycin yielded no formaldehyde. Fried and Stavely (5) have also found that the action of periodic acid on streptomycin B and on dihydrostreptomycin B produces zero and one mole of formaldehyde, respectively.

Sodium metaperiodate was found to be the most suitable reagent for the oxidation of dihydrostreptomycin. Although the action of the salt

is not as rapid as the free periodic acid, there is less danger of hydrolysis with the salt because of the difference in the pH of the solutions. Chromotropic acid (1, 8 Dihydroxynaphthalene-3, 6-Disulfonic Acid) was selected as the best reagent to determine the liberated formaldehyde (6). Attempts to determine the formaldehyde with this reagent in the presence of the other oxidation products were not satisfactory. As a result, it became necessary to distill the formaldehyde from the reaction solution and then determine the formaldehyde in the distillate by means of the chromotropic acid reagent.

PROCEDURE

An aqueous solution of the dihydrostreptomycin salt is prepared to give an estimated 20,000 µg/ml. A 5-ml aliquot of this solution is transferred to a 50-ml glass stoppered flask and 10 ml of an aqueous 0.05M sodium metaperiodate solution added. The oxidation is allowed to proceed overnight at room temperature. After oxidation, a 3-ml aliquot is transferred by means of a pipette to the distilling bulb (Figure 1). An excess of sodium thiosulfate (approximately saturated solution) is added to destroy any remaining periodate. The formaldehyde is then distilled by steam while passing a small stream of air through the system to aid the distillation. Approximately 48 ml of distillate is collected in a 50-ml volumetric flask and made to volume with water. A 1-ml aliquot of the distillate is transferred to a 50-ml volumetric flask and 10 ml of concentrated sulfuric acid is slowly added. One ml of the chromotropic acid reagent (2.5 grams chromotropic acid plus 0.5 gram sodium bisulfite per 100 ml water) is added and the flask is heated in a boiling water bath for 15 minutes. The solution is cooled, distilled water is added with cooling and the solution finally made to 50 ml with distilled water at room temperature. A blank is prepared by adding 1 ml of water, 10 ml of concentrated sulfuric acid, 1 ml of chromotropic acid reagent, heating and making to volume as above. The per cent transmittance of the unknown solution is read in a 1-cm cell in a photoelectric colorimeter, using a 575-mµ filter and setting the instrument at 100 per cent transmittance for the blank solution.

A standard curve is prepared by oxidizing a standard solution of dihydrostreptomycin, distilling aliquots into separate 50-ml flasks and treating the distillates as indicated above with chromotropic acid. The per cent transmittances obtained on the standard solutions are plotted

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

TABLE I

*Comparison of chemical and bioassay of commercial samples of dihydrostreptomycin**

Mfgr.	Type salt	Grams of dihydrostreptomycin per vial	
		Chemical	Bioassay
A	Sulfate	1.10	1.11
	Sulfate	1.10	1.04
B	Hydrochloride	1.10	.99
	Hydrochloride	1.12	1.12
C	Sulfate	1.06	1.04
	Sulfate	.90	1.05
D	Sulfate	1.06	1.03
	Sulfate	1.05	1.08
E	Sulfate	1.23	1.06
	Sulfate	1.05	1.00

* All of the above samples were labeled to contain one gram of dihydrostreptomycin per vial.

as the ordinate on semi-log paper against the concentrations in micrograms of the dihydrostreptomycin standard as the abscissa. A straight line relationship is obtained. The per cent transmittance of the unknown is located on the curve and the corresponding concentration of dihydrostreptomycin read off the graph.

Table I shows the values obtained when ten commercial samples were analyzed by this procedure as compared to the turbidimetric bioassay method. The values indicate a good agreement between the two methods. The standard dihydrostreptomycin sulfate² used in this study was a relatively pure sample as determined by chemical and biological assay.

A series of streptomycin samples were analyzed for their dihydrostreptomycin content by this

² Supplied through the courtesy of Chas. Pfizer & Co., Inc.

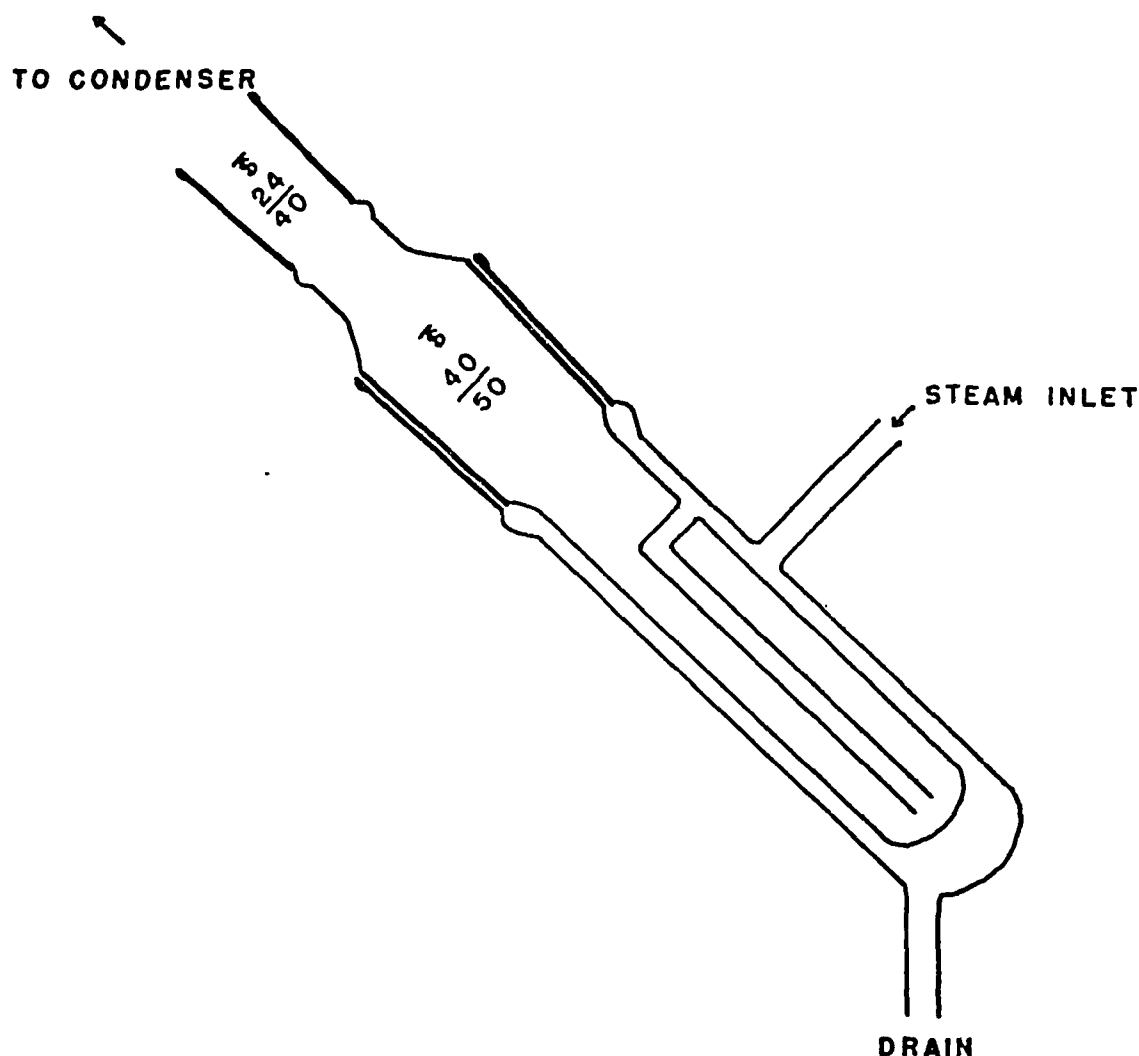


FIG. 1. DISTILLATION BULB FOR DISTILLING FORMALDEHYDE WITH STEAM

procedure. Two samples (both from the same manufacturer) showed 10 and 13 per cent dihydrostreptomycin while the others showed less than 6 per cent. It is possible that impurities capable of reacting with periodate to give formaldehyde are responsible for some of the apparent dihydrostreptomycin content of these commercial streptomycin samples. The FDA streptomycin sulfate working standard showed less than 3 per cent dihydrostreptomycin by the above method. A relatively pure sample of streptomycin B³ also showed less than 3 per cent dihydrostreptomycin as determined by this procedure. The data obtained during this study indicate the proposed chemical method is reliable for the analysis of relatively pure dihydrostreptomycin samples.

SUMMARY

Dihydrostreptomycin may be quantitatively determined by the colorimetric measurement of the formaldehyde liberated after oxidation with periodate.

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³ Supplied through the courtesy of Heyden Chemical Corporation.

INVESTIGATIONS ON THE STREPTOMYCINS¹

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Studies *in vitro* and *in vivo* with pure preparations of streptomycin, dihydrostreptomycin, mannosidostreptomycin and dihydromannosidostreptomycin have been continued. Unfortunately, up to the present only small amounts of the two latter antibiotics have been available in the pure form and for this reason *in vivo* studies have been carried out only with the two former.

Careful studies of the *in vitro* activity of these four streptomycins on nine species and 16 strains have been reported elsewhere (1). In general a pattern of activity was established which showed approximately equal activity of streptomycin and dihydrostreptomycin in terms of minimal inhibiting concentrations (M.I.C.) of antibiotic. Mannosidostreptomycin and dihydromannosidostreptomycin also showed M.I.C.'s equal to each other but significantly higher than streptomycin and dihydrostreptomycin, *i.e.* the mannosido-compounds were less active. The chief exceptions to this pattern were with *S. typhosa* and *S. schottmülleri* where dihydrostreptomycin was much less active than streptomycin but the mannosido-compounds showed activity similar to that of streptomycin.

Further *in vitro* studies have confirmed this general pattern of activity. In Table I are shown the data for nine species of which two have not been reported previously—*H. influenzae* and *D. granulomatis*. They follow the usual pattern. Of note is the fact that in both cases the dihydro compounds are slightly but significantly more active, and that *D. granulomatis* is by far the most sensitive strain we have tested so far.

In Table II are shown the *in vitro* results with 12 strains of seven species of *Salmonellae* and *Shigellae*. Although the two species of *Shigella* fit in with the pattern discussed above and shown by the strains in Table I, the *Salmonellae* are

atypical as already indicated in the previous publication (1). Moreover, there are three distinct groupings with the *Salmonellae*. All strains show a higher resistance to dihydrostreptomycin than to streptomycin, this being least marked with *S. enteritidis* where the activity of the dihydro compound almost equals that of streptomycin while it is only one-third to one-fourth as active as the latter compound for the other species. The three distinct groupings appear when the activity of the mannosidostreptomycins is considered. With *S. enteritidis* the pattern resembles that of *Shigella* and the other microorganisms. With all strains of *S. typhosa* and *S. schottmülleri* the mannosidostreptomycins have an activity in terms of M.I.C. approximately equal to that of streptomycin while with *S. pullorum* and *S. gallinarum* the mannosidostreptomycins are more than twice as active. This activity of the mannosidostreptomycins against the *Salmonellae* is of particular interest in consideration of the mode of action of the different streptomycins. Although in the case of most organisms it might be hypothesized that the mannosidostreptomycins were only active after conversion to streptomycin, and that their lesser activity was due to incomplete conversion by the organisms involved, such cannot be the case with their activity on *S. pullorum* and *S. gallinarum* and the whole hypothesis must thus be put in question.

Unfortunately, at the present time insufficient amounts of the pure mannosidostreptomycins are available for *in vivo* tests. At this time further *in vivo* experiments with streptomycin and dihydrostreptomycin will be described and the results of different dosage schedules with these two compounds will be evaluated.

In Table III are seen the composite results of several tests comparing the activity of streptomycin and dihydrostreptomycin against *Rickettsia microti*—the vole rickettsia of Baker, in the egg. It will be noted that dihydrostreptomycin is twice as active as streptomycin itself. This finding is the reverse of those of Smadel, Jackson and Gauld (2) with other species. They found di-

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

TABLE I
In vitro activities of pure streptomycins against various organisms

Test organism	Minimal inhibiting concentrations*			
	Streptomycin	Dihydro-streptomycin	Mannosido-streptomycin	Dihydromannosido-streptomycin
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
<i>Clebsiella pneumoniae</i> (ATCC 9997)	1.76	1.76	6.39	6.59
<i>Errobacter aerogenes</i> (ATCC 129)	2.71	3.27	10.8	11.1
<i>Escherichia coli</i> (D-56)	6.05	6.79	24.8	23.8
<i>Emophilus influenzae</i> , Type B (D-68)	2.30	1.53	8.53	5.53
<i>Donovania granulomatis</i> (B-44A)	0.167	0.151	0.595	0.446
<i>Staphylococcus aureus</i> (209-P)	0.828	1.39	5.64	7.77
<i>Streptococcus pyogenes</i> (C-203)	11.7	15.9	82.9	87.9
<i>Brucella abortus</i> (Huddleson 1119 avirulent)	0.816	0.738	2.93	2.53
<i>Mycobacterium tuberculosis</i> :				
H-37Rv	2.0	2.2	5.5	6.5
Ravenel	0.58	0.62	2.5	2.2
BCG	0.52	0.55	1.9	1.7
N.†	0.54	0.56	2.5	2.1
T.†	0.55	0.54	2.2	2.0
P.†	0.62	0.85	2.3	2.2
O'D.†	0.63	0.75	2.3	2.6
K.†	1.0	1.7	3.9	3.9

* All figures given in terms of weight of the trihydrochlorides.

† Strains of *M. tuberculosis* freshly isolated from human cases.

hydrostreptomycin less than half as active as streptomycin in the egg against *R. rickettsii* and *R. akari*.

In seeking to obtain definitive data on the usefulness of different dose schedules both acute and chronic infections have been employed. In Table IV are shown the results with one of the acute infections used, namely, that with *S. schottmülleri*. It will be noted that in all cases a single daily dose schedule is better (*i.e.* the CD_{50} is less) than is a three dose schedule. This is more marked where

a small bacterial inoculum is used but even with larger inocula the results are significant. In this table one should draw attention to a phenomenon mentioned before (1), namely, that dihydrostreptomycin is more active *in vivo* (better than half as active as streptomycin) than might be expected from its *in vitro* activity (see Table II).

With more chronic infections there is no actual advantage to the single dose schedule. Instead, one, two, or three dose schedules all give the same response. This is shown in Tables V and

TABLE II
In vitro activities of pure streptomycins against various organisms

Test organism	Minimal inhibiting concentrations*			
	Streptomycin	Dihydro-streptomycin	Mannosido-streptomycin	Dihydromannosido-streptomycin
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
<i>Salmonella typhosa</i> (D-15)	12.2	51.0	12.4	12.9
<i>Salmonella typhosa</i> (D-14)	9.73	34.3	9.14	9.16
<i>Salmonella typhosa</i> (D-59 rough)	6.76	23.3	8.06	7.09
<i>Salmonella schottmülleri</i> (D-51)	10.1	36.5	14.3	14.4
<i>Salmonella enteritidis</i> (D-61)	4.14	5.50	12.7	13.6
<i>Salmonella enteritidis</i> (D-125)	3.26	5.76	12.1	14.0
<i>Salmonella enteritidis</i> (D-124)	1.71	2.25	7.09	6.64
<i>Salmonella enteritidis</i> (D-126)	4.21	5.72	15.6	16.5
<i>Salmonella pullorum</i> (D-123)	26.5	90.1	12.5	14.7
<i>Salmonella gallinarum</i> (D-122)	29.1	116	13.3	13.8
<i>Shigella sonnei</i> (D-116)	7.42	8.52	30.6	30.3
<i>Shigella dysenteriae</i> (D-105)	6.26	5.82	27.2	27.1

* All figures given in terms of weight of the trihydrochlorides.

TABLE III

Comparison of pure streptomycin and dihydrostreptomycin in vole rickettsial infections in embryonated chick eggs

	No. of tests	Total no. of eggs	Dead/Total	% Dead
Controls 10 ⁻² *	5	34	30/34	88.2
Controls 10 ⁻³	5	40	25/40	62.5
Controls 10 ⁻⁴	5	40	22/40	55.
Streptomycin				
0.001 mg/egg†	1	6	5/6	83.3
0.004 mg/egg†	5	34	24/34	70.6
0.016 mg/egg†	5	34	18/34	53.0
0.064 mg/egg†	5	34	11/34	32.4
0.25 mg/egg†	5	33	4/33	12.2
1.00 mg/egg†	3	22	0/22	0

CD₅₀ = 0.02 mg/egg

Dihydrostreptomycin				
0.001 mg/egg†	1	6	5/6	83.3
0.004 mg/egg†	5	34	21/34	61.7
0.016 mg/egg†	5	34	15/34	44.2
0.064 mg/egg†	5	34	5/34	14.7
0.25 mg/egg†	5	34	4/34	11.8
1.00 mg/egg†	3	22	1/22	4.5

CD₅₀ = 0.01 mg/egg

* 10⁻² refers to dilution of yolk sac suspension used as inoculum. All treated eggs were inoculated with the 10⁻² dilution.

† Dosage given in terms of weight of pure trihydrochloride.

VI where a smaller and larger total daily dosage are compared in a standardized tuberculous infection in mice. It will be seen that when the average survival time in days is compared there is no significant difference in one type of dosage

TABLE IV

Effect of dosage schedule on streptomycin and dihydrostreptomycin action in *Salmonella schottmülleri* infection in mice

Antibiotic preparation	No. of tests	Average inoculum* bacterial cells/mouse	Therapy schedule	CD ₅₀ mg/kg
Streptomycin (650 u/mg)	5	60	Single dose	4.2
Streptomycin (650 u/mg)	5	60	3 doses in 1 day	8.6
Streptomycin (850 u/mg)	3	1100	Single dose	24.0
Streptomycin (850 u/mg)	3	1100	3 doses in 1 day	38.5
Dihydrostreptomycin (550 u/mg)	3	1100	Single dose	40.0
Dihydrostreptomycin (550 u/mg)	3	1100	3 doses in 1 day	69.0

* One to two bacterial cells per mouse were lethal to all control mice.

TABLE V

Effect of schedule of treatment with streptomycin* in mice infected with *Mycobacterium tuberculosis* (Ravenel)

No. of mice	Total daily dose† Units/kg/Day	Schedule	Duration of treatment (days)	Average survival time (days)	% Surviving longer than 34 days
8	None (Controls)	—	—	19.8	0
8	10,000	3333 t.i.d.	7	21.9	0
8	10,000	5000 b.i.d.	7	23.5	0
8	10,000	Single dose	7	22.8	0
8	10,000	3333 t.i.d.	14	26.0	0
8	10,000	5000 b.i.d.	14	25.6	0
8	10,000	Single dose	14	25.9	0
8	10,000	3333 t.i.d.	21	27.6	12.5
8	10,000	5000 b.i.d.	21	28.5	12.5
8	10,000	Single dose	21	27.5	0

* Partially purified streptomycin, 400 units/mg.

† Treatment given via the subcutaneous route.

schedule over another whether the total daily dose is 10,000 or 50,000 units per kg or whether treatment is maintained for seven, 14 or 21 days.

In general, it can be said then that no benefit can be expected in setting up any standardized test for the streptomycins with a dosage schedule using greater frequency than one dose a day. One would presume that the same would hold for the mannosidostreptomycins when they become available.

TABLE VI

Effect of schedule of treatment with streptomycin* in mice infected with *Mycobacterium tuberculosis* (Ravenel)

No. of mice	Total daily dose† Units/kg/Day	Schedule	Duration of treatment (days)	Average survival time (days)	% Surviving longer than 34 days
8	None (Controls)	—	—	19.8	0
7	50,000	16,667 t.i.d.	7	31.7	14.3
7	50,000	25,000 b.i.d.	7	28.7	14.3
8	50,000	Single dose	7	30.1	28.6
8	50,000	16,667 t.i.d.	14	37.4	87.5
8	50,000	25,000 b.i.d.	14	37.9	87.5
8	50,000	Single dose	14	40.5	75.0
8	50,000	16,667 t.i.d.	21	52.0	100
8	50,000	25,000 b.i.d.	21	59.0	100
8	50,000	Single dose	21	59.4†	100

* Partially purified streptomycin, 400 units/mg.

† Treatment given via the subcutaneous route.

† One mouse survived through 118 days, at which time it was sacrificed.

SUMMARY

In vitro studies continue to reveal unique behavior on the part of the various strains of *Salmonellae* in their response to the four streptomycins. Where with most other species the activity of dihydrostreptomycin equals that of streptomycin, in this genus all strains tested are less sensitive to the former than to the latter, all comparisons being made on a weight basis. On the other hand, for most genera studied the mannosidostreptomycins are less active than is streptomycin. In contrast to this for the *Salmonellae*, the mannosido compounds are often as active as streptomycin, and for two species are even more active than the latter. The characteristics of dihydrostreptomycin mentioned above do not hold for dihydromannosidostreptomycin which approximately equals mannosidostreptomycin in activity for every species tested thus far *in vitro*.

In vivo studies in acute as well as in chronic experimental infections show that streptomycin or dihydrostreptomycin given in a single dose daily gives results as good as, or better than, those obtained by giving two to three divided doses daily. Against *S. schottmülleri* dihydrostreptomycin is more active than might be expected from the *in vitro* findings. In embryonated eggs infected with *Rickettsia microti* dihydrostreptomycin is somewhat more active than streptomycin.

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THE EFFECT OF STREPTOMYCIN DERIVATIVES ON STREPTOMYCIN-DEPENDENT AND -RESISTANT STRAINS OF BACTERIA¹

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Several authors (1, 2) have recently demonstrated the value of streptomycin-dependent strains of bacteria for the identification of streptomycin-producing organisms. The occurrence of streptomycin-dependent mutants of various bacteria was first demonstrated by Miller and Bohnhoff (3, 4). The streptomycin-dependent mutants were found in cultures which also contained streptomycin-resistant strains of the usual type.

In the case of the streptomycin-dependent strains, these organisms, according to Iverson and Waksman, apparently utilize streptomycin as a true growth factor. The authors stated that streptomycin is not used as a substrate or a nutrient since no destruction of streptomycin occurred in media in which the streptomycin-dependent strains were grown.

Accepting the suggestion that streptomycin represents a true growth factor for the mutant forms discussed above, it was of interest to determine whether any synthetic streptomycin derivatives could act either as anti-metabolites vis-à-vis the "growth factor" streptomycin or possibly as substitute growth factors for a streptomycin-dependent organism. It was also thought of interest to extend the study to include the effects of various streptomycin derivatives on streptomycin-resistant bacteria, as well.

Various streptomycin derivatives were therefore tested for antistreptomycin or streptomycin-like activity. The derivatives examined included dihydrostreptomycin (5) and certain N-substituted straight chain alkyl streptomycylamines first described by Winsten (6).

In testing different preparations of the streptomycylamine derivatives, it was necessary to insure that each derivative was tested for its effects free of any residual unchanged streptomycin or dihydrostreptomycin formed during the course of the

synthesis of the streptomycylamine derivatives by catalytic hydrogenation of the corresponding Schiff bases. This could be most conveniently done by use of the paper chromatographic method of Winsten and Eigen (7).

METHODS

In carrying out the present study a solution of each preparation of a derivative (or derivatives) was first subjected to paper chromatographic separation. The resulting paper chromatograms were then laid on agar seeded with *E. coli* Strain E 158, a streptomycin-dependent strain obtained through the courtesy of Dr. R. J. Vanderlinde.

A desired amount of streptomycin was included in the Difco nutrient agar, in order to insure sufficient background growth of *E. coli* E 158 where this was desired. After incubation the substances being tested either caused enhanced zones of growth or zones of inhibition, seen along the loci of the strip chromatograms. The zones of growth were seen against the lighter background growth caused by the streptomycin in the medium. The zones of inhibition were seen as on usual plates against the background bacterial growth. Indeed it sometimes occurred that a locus of a chromatogram exhibited both a zone of growth and a zone of inhibition. This situation occurred whenever a preparation of a derivative which was an inhibitor also contained unchanged streptomycin or dihydrostreptomycin which caused zones of growth. A schematic drawing of such a chromatogram is shown in Figure 1 for the case of an N-n-hexylstreptomycylamine preparation.

RESULTS

The results of a series of experiments in which dihydrostreptomycin, streptomycin and different preparations of the N-substituted straight chain alkylstreptomycylamine derivatives were first subjected to paper chromatography and then tested against *E. coli* E 158 are given in Table I.

From the table it is apparent that streptomycin and dihydrostreptomycin cause zones of growth of *E. coli* E 158, when the agar contains 10 γ /ml of streptomycin. At a level of 100 γ /ml of streptomycin the background growth was quite heavy, thus obscuring zones of growth caused by these two substances.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

Rake (8) has also reported that organisms which require streptomycin for growth can also use dihydrostreptomycin for the same purpose.

It is also evident from Table I that in the presence of 10 γ of streptomycin per ml of agar, N-n-

Site of application
of test sample

Zone of growth due
to residual strepto-
mycin in preparation

$$R_F = 0.25$$

Zone of inhibition
due to N-n-hexyl-
streptomycylamine

$$R_F = 0.77$$

Solvent front

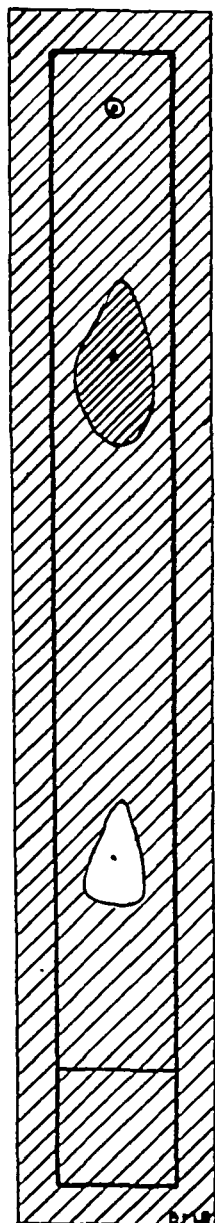


FIG. 1. SCHEMATIC DIAGRAM OF LOCUS OF A CHROMATOGRAM ON AN AGAR PLATE SEEDED WITH STREPTOMYCIN-DEPENDENT STRAIN *E. coli* E 158

The more widely spaced diagonal lines represent the lighter background bacterial growth caused by the presence of 10 γ of streptomycin per ml of agar. The chromatogram was developed for 18 hours on Whatman No. 1 paper using as a solvent wet butanol-2% piperidine-2% p-toluenesulfonic acid.

TABLE I

The effect of streptomycin and its derivatives on E. coli E158, seeded into agar containing streptomycin

G—indicates a zone of growth seen against the background bacterial growth. I—represents a zone of inhibition. N—represents neither a zone of growth nor of inhibition.

Substance studied*	Zones found	
	Amount of streptomycin in agar	
	10 γ per ml	100 γ per ml
Streptomycin	G	N
Dihydrostreptomycin	G	N
N-n-propylstreptomycylamine	N	N
N-n-butylstreptomycylamine	I	N
N-n-hexylstreptomycylamine	I	I, N†
N-n-octylstreptomycylamine	I	I
N-n-decylstreptomycylamine	I	I
N-n-dodecylstreptomycylamine	I	

* 10-microliter samples of 1–6% solutions of the various preparations were chromatographed on Whatman No. 1 or No. 4 paper for 18 hours using butanol-2% piperidine-2% p-toluenesulfonic acid as the developing solvent.

† The N-n-hexyl derivative in one experiment caused a hazy zone of inhibition. In a second experiment it caused no zone.

propylstreptomycylamine did not cause a zone of growth or of inhibition. The N-n-butyl derivative and higher homologues caused zones of inhibition. In the presence of 100 γ of streptomycin per ml of agar, the N-n-propyl and N-n-butyl derivatives caused no zones of inhibition whereas the higher homologues did so. The N-n-hexyl derivative appears to be a borderline case; in one experiment a zone of inhibition hazy in character was observed whereas in a second experiment no zone of inhibition was observed.

From these findings for the streptomycin-dependent *E. coli* E 158, there would appear to be an interesting metabolite-antimetabolite relationship between streptomycin and various N-substituted streptomycylamine derivatives which is some function of the length of the substituent side chain.

The above studies have been extended to a streptomycin-dependent strain of *S. aureus*. Starting with *S. aureus* SM, there was isolated an organism which grew readily in broth containing 1000 γ /ml of streptomycin. The culture was found to contain organisms which required streptomycin for growth. It was possible by subculture in streptomycin-free media to obtain organisms which grew in the absence of and were resistant to streptomycin.



FIG. 2. LOCI OF CHROMATOGRAMS ON AGAR PLATE SEEDED WITH *S. aureus* H. A 10-microliter sample of a 3% solution of each preparation was chromatographed for 18 hours at room temperature using wet n-butanol-2% piperidine-2% p-toluenesulfonic acid as the developing solvent: Strip 1, N-n-propylstreptomycylamine preparation on Whatman No. 4 paper; Strip 2, N-n-butylstreptomycylamine preparation on Whatman No. 1 paper; Strip 3, N-n-hexylstreptomycylamine preparation on Whatman No. 1 paper (see text for discussion of results).



FIG. 3. LOCI OF CHROMATOGRAMS ON AGAR PLATE SEEDIED WITH A HIGHLY STREPTOMYCIN-RESISTANT STRAIN OF *S. aureus*

A 10-microliter sample of a 3% solution of each preparation was chromatographed 18 hours at room temperature using wet n-butanol-2% piperidine-2% p-toluenesulfonic acid as the developing solvent: Strip 1, N-n-propylstreptomycylamine preparation on Whatman No. 4 paper; Strip 2, N-n-butylstreptomycylamine preparation on Whatman No. 1 paper; Strip 3, N-n-hexylstreptomycylamine preparation on Whatman No. 1 paper (see text for discussion of results).

TABLE II

The effect of streptomycin and its derivatives on a mixed culture of S. aureus containing both streptomycin-dependent and -resistant strains of bacteria

G—indicates a zone of growth seen against the background bacterial growth. I—represents a zone of inhibition. 30 γ per ml of streptomycin was included in the agar.

Substance studied*	Zones found
Streptomycin	G
Dihydrostreptomycin	G
N-n-propylstreptomycylamine	G
N-n-butylstreptomycylamine	G
N-n-hexylstreptomycylamine	G
N-n-octylstreptomycylamine	I
N-n-decylstreptomycylamine	I
N-n-dodecylstreptomycylamine	I

* 10-microliter samples of 1–6% solutions of the various preparations were chromatographed on Whatman No. 1 or No. 4 paper for 18 hours at room temperature using butanol-2% piperidine-2% p-toluenesulfonic acid as the developing solvent.

It is therefore likely that the culture containing the streptomycin-dependent organisms also contained a resistant strain as well. Using this mixed culture seeded into agar containing 30 γ /ml of streptomycin to promote the background growth of the dependent strain, the results recorded in Table II were obtained in a manner similar to that described earlier for *E. coli* E 158. Inspection of the data shows that streptomycin, dihydrostreptomycin and the shorter chain streptomycylamine derivatives up to hexyl caused zones of growth. The longer chain length derivatives caused zones of inhibition.

It is interesting to compare these results for a streptomycin-dependent strain of *S. aureus* with those obtained for *E. coli* E 158. It will be recalled that in the case of *E. coli* the N-n-propylstreptomycylamine derivative caused neither a zone of growth or of inhibition. On the other hand the streptomycin-dependent strain of *S. aureus* can utilize not only the N-n-propyl derivative for growth but the N-n-butyl and N-n-hexyl derivatives as well, both of which two latter derivatives inhibit *E. coli* E 158.

It would therefore appear that there are differences of major importance between different streptomycin-dependent organisms with regard to their response to streptomycylamine derivatives.

In the light of the above findings it was of interest to extend these studies to a streptomycin-resistant organism. It is well known that an organism resistant to streptomycin is also resistant to dihydrostreptomycin. It was anticipated that

some streptomycin-resistant bacteria might be sensitive to various members of the N-substituted streptomycylamine family of antibiotics. Such appears to be the case for at least one highly streptomycin-resistant strain of *S. aureus*.

Preparations of N-n-propyl-, N-n-butyl- and N-n-hexylstreptomycylamine were first tested, using the paper chromatographic technique, against the parent strain *S. aureus* H from which a highly streptomycin-resistant strain was derived. The results of this experiment appear in Figure 2.

Inspection of Figure 2 reveals that the N-n-propyl preparation contained the faster moving derivative itself, some dihydrostreptomycin, and two slow moving antibiotics of unknown structure which formed a curious doublet zone of inhibition. The zones of inhibition appear somewhat smeared; this was probably due to the use of Whatman No. 4 paper, a chromatographically very fast paper.

Figure 2 also reveals that the N-n-butylstreptomycylamine preparation, chromatographed on the slower Whatman No. 1 paper, exhibited a zone due to the fast moving N-n-butyl derivative itself as well as two small zones of inhibition due to dihydrostreptomycin (the slowest antibiotic in the preparation) and streptomycin.

Chromatographic analyses of the N-n-hexylstreptomycylamine preparation used in this experiment reveal in Figure 2 that the particular preparation used contained only a trace of the fast moving N-n-hexyl derivative itself. The main zone of inhibition observed in Figure 2 for this preparation is due to streptomycin itself. The N-n-hexyl preparation therefore merely serves to provide streptomycin as a control substance in the plate chromatographic test of Figure 2.

In Figure 3 are shown the results of chromatographing identical amounts of the N-n-propyl, N-n-butyl, N-n-hexylstreptomycylamine preparations studied in Figure 2, using a highly streptomycin-resistant strain of *S. aureus* derived from *S. aureus* H as the test organism. From the figure it is evident that only the N-n-propyl- and N-n-butylstreptomycylamine derivatives caused readily visible zones of inhibition. If one examines the chromatogram of the N-n-hexyl preparation, the large residual streptomycin zone present in Figure 2 for the parent *S. aureus* H is now completely absent for the highly resistant daughter strain of

S. aureus derived from H. A trace zone due to the N-hexyl derivative itself can be seen on careful inspection of Figure 3 at about the same point as in Figure 2. In a separate experiment a second preparation of N-n-hexylstreptomycylamine containing a larger amount of the derivative as is evident in Figure 1 caused a larger zone of inhibition at a comparable point on the chromatogram. The zone was present when either *S. aureus* H or its streptomycin-resistant offspring was used as the test organism.

In still other experiments the N-n-octyl- and the N-n-decylstreptomycylamine derivatives caused zones of inhibition when using the resistant *S. aureus* as the test organism. These zones however were turbid or hazy, suggesting merely a retarded growth of the organism rather than complete inhibition.

The findings in Figure 3, wherein a highly streptomycin-resistant strain of *S. aureus* was inhibited by the N-n-propyl and the N-n-butyl derivatives, were confirmed in serial dilution tube assays. Whereas the resistant strain derived from *S. aureus* H was not inhibited by as much as 10,000 γ per ml of streptomycin, it was completely inhibited in a 24 hour test by 5 γ per ml of the N-n-propyl derivative or 4 γ per ml of the N-n-butyl derivative. 25 γ per ml of the N-n-decyl derivative were needed for inhibition while the N-n-octyl derivative failed to inhibit as 100 γ per ml (the highest amount tried). *S. aureus* H itself was inhibited by 1 γ per ml of streptomycin, 16 γ per ml (or less) of the N-n-propyl derivative and 63 γ per ml of the N-n-butyl derivative.

DISCUSSION

The above findings suggest that it may be possible to obtain N-substituted streptomycylamine derivatives which are active against streptomycin-resistant strains of bacteria. The results indicate however that a derivative active against one streptomycin-resistant organism may not be active against another. This is based on the findings reported earlier in this paper where the N-n-butyl derivative could be used for growth by one streptomycin-dependent organism (an *S. aureus*), but caused inhibition of another streptomycin-depend-

ent strain (*E. coli* E 158) by antagonizing the growth promoting influence of streptomycin.

The results reported in this paper suggest that it may be possible to obtain a particular streptomycin derivative active against a particular streptomycin-resistant strain of bacteria. Further studies along this line will be reported at a later date.

SUMMARY

1. A streptomycin-dependent strain of *E. coli* was inhibited by various members of an homologous series of N-substituted alkyl streptomycylamines, in the presence of streptomycin.
2. A streptomycin-dependent strain of *S. aureus* was found capable of using N-n-propyl-, N-n-butyl-, and N-n-hexylstreptomycylamine for growth, replacing the need for streptomycin. Higher homologues inhibited the growth of the organism in the presence of streptomycin.
3. A streptomycin-resistant strain of *S. aureus* was inhibited markedly by N-n-propylstreptomycylamine, N-n-butylstreptomycylamine, and N-n-decylstreptomycylamine.

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THE DEVELOPMENT OF RESISTANCE OF *SALMONELLA TYPHOSA* TO THE FATTY ACID SALTS OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN¹

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Since the discovery of streptomycin by Waksman (1) there have been two major problems involved in its clinical use. Streptomycin was shown early to be inherently toxic, causing eighth-nerve damage in relatively low doses, particularly when given over a long period of time. In addition, all organisms sensitive to streptomycin have been shown to develop resistance to it rapidly, and the resistance is not only demonstrable *in vitro* but *in vivo* as well. The development of dihydrostreptomycin has partially answered the problem concerning streptomycin toxicity; however, organisms become resistant to dihydrostreptomycin with the same facility and to a similar degree. In the present study, an attempt has been made to develop salts of streptomycin and dihydrostreptomycin to which organisms either do not develop a resistance or develop resistance less readily than they do to the salts now available.

It has been known for some time that fatty acids or derivatives of fatty acids may influence markedly the development and severity of experimental tuberculosis in animals. Negre, Berthelot, and Bretey (2) were able to retard the appearance of tuberculosis lesions in guinea pigs by the injection of the ethyl esters of a series of fatty acids. Recently, Hedgecock (3) has reported that the oral administration of a diet containing the total fatty acids of coconuts also retarded the development of experimental tuberculosis in animals. Some fats and fatty acids, on the other hand, may enhance the development of experimental tuberculosis in animals. Since streptomycin is a base and has the ability to combine with fatty acids, it was thought worthwhile to make compounds of this nature for test purposes.

The fatty acid salts studied, the caprylate, undecylate, and propionate salts of both streptomycin and

dihydrostreptomycin were prepared by electro dialyzing the sulfates or hydrochlorides to the corresponding bases (4). The streptomycin base collected at the cathode was then titrated to a pH of 6.5-7 with the respective fatty acid. For the more insoluble fatty acids this titration was accomplished by the addition of a few drops of ethanol followed by slow addition of the fatty acid with periodic agitation in a high speed Waring Blendor as the desired pH was approached. The material was finally filtered to remove excess streptomycin fatty acid salt, leaving a saturated filtrate.

The fatty acid salts prepared by this method were tested for inhibition of the growth of 56 species of bacteria representing 17 genera. Included as controls were streptomycin calcium chloride complex and dihydrostreptomycin sulfate. The medium used was yeast beef broth (Difco). The ranges of the minimal inhibitory concentrations for the species in the various genera are tabulated in Table I. It can be readily seen that, as previously reported by Rake *et al.* (5), various members of the genus *Salmonella* are more resistant to dihydrostreptomycin than to streptomycin. The species of mycobacteria tested, *smegmatis*, *phlei*, and *avian*, all show a high sensitivity to the salts under study. In some of the genera there exists a wide variation in the sensitivity of the individual species to the same salt. It is apparent also that there is a considerable but less marked difference in the sensitivity of the same species to the different compounds. These differences indicate that the anionic portion of the molecule may affect materially the activity of streptomycin and dihydrostreptomycin against certain strains of bacteria. This is strikingly shown in the next experiment in which all eight salts were tested for their ability to prevent the increase in resistance of a strain of *S. typhosa* shown by previous studies (6) to have the ability of rapidly increasing in resistance to streptomycin. Serial dilutions of the eight salts were made in standard methods broth, pH 7.8 to 8. All dilutions were then inoculated with standard methods broth containing 1% of a 24-hour broth culture of *S. typhosa*. These were then in-

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TABLE I

STREPTOMYCIN COMPOUNDS: RANGE OF CONCENTRATION IN Mcg/ml. NECESSARY TO INHIBIT VARIOUS MICRO-ORGANISMS

GENUS	NO.	STREPTOMYCIN				DIHYDROSTREPTOMYCIN			
		COMPLEX	UNDECYLENATE	CAPRYLATE	PROPIONATE	SULFATE	UNDECYLENATE	CAPRYLATE	PROPIONATE
MYCOBACTERIA	3	.04-.15	.15-.37	.08-.15	.08-.37	.15-.37	.08-.15	.08-.37	.15-.37
CORYNOBACTERIUM	1	.75	.08	.08	.15	.37	.15	.625	.37
NEISSERIA	1	.75	.75	.75	.37	.37	.75	.75	.75
BACILLI	12	15-250	37-200	15-100	75-50	75-250	37-100	15-100	37-100
AEROBACTOR	1	12.5	6.25	3.12	25	6.25	25	3.12	12.5
ESCHERICHIA	4	25	25	25	12.5-25	25-50	25	12.5-25	12.5-25
SALMONELLA	7	25-100	25-100	25-100	25-100	200-400	50-400	50-200	100-400
SHIGELLA	1	12.5	6.25	12.5	12.5	12.5	12.5	6.25	12.5
STREPTOCOCCUS	2	62-125	125-100	125-100	62-200	15-125	125-100	125-100	125-200
MICROCOCCUS	7	625-50	125-25	15-50	15-50	3.12-50	75-50	75-50	75-100
KLEBSIELLA	2	.15-.15	75-12.5	75-25	37-25	15-75	15-12.5	37-12.5	75-12.5
PSEUDOMONAS	2	100	100	100	100	100-200	100	100	100
SARCINA	2	.37-1.5	.04-.75	.37-.75	.37-3.12	.15-1.5	.15	.08-3.12	.15-1.5
SERRATIA	3	312-500	125-100	125-50	50-200	250-500	625-200	125-200	125-400
BRUCELLA	2	75-100	75-100	75-100	75-200	75-200	37-200	37-200	75-200
LEUCONOSTOC	1	3.12	3.12	3.12	.75	125	3.12	3.12	.75
CLOSTRIDIUM	4	50-250	50-200	50-200	50-200	3.12-250	100	25-100	25-200

cubated overnight at 37°C and the tube containing the largest concentration of the salt which showed growth was used as the inoculum for the next series of dilutions. This was continued for 22 transfers. In Table II is shown the initial sensitivity of *S. typhosa* to the eight salts, and the sensitivity obtained on the 22nd transfer.

It can be seen that the strain of *S. typhosa* used in these studies increased in resistance to streptomycin calcium chloride complex and to dihydrostreptomycin sulfate to a far greater extent than to the fatty acid salts used. The increase in resistance to streptomycin caprylate and undecylenate was less than with any of the other salts tested.

In order to determine the specificity of the resistance developed, the strains of *S. typhosa* made resistant to the fatty acid salts of streptomycin and to streptomycin calcium chloride complex were tested for their resistance to each of the other salts. For comparative purposes, streptomycin sulfate and streptomycin hydrochloride were included. The results are shown in Table III.

It will be noted that when a strain of *S. typhosa* is made resistant to 5000 µg/ml of the calcium chloride complex salt of streptomycin, its resistance to the fatty acid salts was found to be considerably less. In the case of the undecylenate and caprylate salts, this resistance was less than 100

µg/ml. The propionate salt, which incidentally is the most soluble of the three fatty acid salts studied, caused inhibition of growth of *S. typhosa* at a concentration of 187 µg/ml, a somewhat higher level. It was necessary, however, to utilize 625 µg/ml of streptomycin sulfate and 5000 µg/ml of the streptomycin hydrochloride to cause inhibition of *S. typhosa* which had been made resistant to 5000 µg/ml of the calcium chloride complex salt of streptomycin. Except in the case of the propionate salt, whenever *S. typhosa* was made resistant to

TABLE II
Increase in resistance of *S. Typhosa* to Fatty acid salts of streptomycin and dihydrostreptomycin

Streptomycin salt	Minimum inhibition conc. in µg/ml*		
	Initial	After 22 transfers	Increased resistance ratio
Caprylate	0.07	25	356
Propionate	0.07	531	7,600
Undecylenate	0.07	62	900
CaCl ₂	0.07	5,000	71,000
Dihydro-streptomycin salt	Initial	After 22 transfers	Increased resistance ratio
Caprylate	0.62	625	1,000
Propionate	0.62	1,500	2,420
Undecylenate	0.31	437	700
Sulfate	0.62	5,000	8,000

* Standard Methods Broth pH 7.8-8 used throughout.

TABLE III

EFFECT OF STREPTOMYCIN SALTS ON RESISTANT *S. TYPHOSA*

STREPTOMYCIN SALT EMPLOYED	MINIMUM CONCENTRATION IN Mcg/ml. NECESSARY TO INHIBIT <i>SALMONELLA TYPHOSA</i> MADE RESISTANT TO:-			
	5000mcg/ml. CaCl ₂ COMPLEX	30mcg/ml. UNDECYLENATE	80mcg/ml. CAPRYLATE	400mcg/ml. PROPIONATE
CaCl ₂ COMPLEX		625	625	312
UNDECYLENATE	62		31	62
CAPRYLATE	94	78		312
PROPIONATE	187	110	110	
SULFATE	625	312	312	312
HYDROCHLORIDE	5000	625	625	312

the fatty acid salts these organisms were always found to be considerably more resistant to the streptomycin salts commercially available. It is of interest that an organism made resistant to 5000 $\mu\text{g}/\text{ml}$ of the calcium chloride complex salt of streptomycin, although showing the same resistance to streptomycin hydrochloride, was found to have a resistance to only 625 $\mu\text{g}/\text{ml}$ of streptomycin sulfate. These results, coupled with those obtained with the fatty acid salts, support the conclusion that the anionic portion of the streptomycin and dihydrostreptomycin molecules may markedly influence the development of resistance of organisms to these drugs.

In order to determine the acute intravenous toxicity of the six fatty acid salts under study,

the LD₅₀ of each was determined in 20-gm. mice. For control purposes, the calcium chloride complex salts of streptomycin and dihydrostreptomycin sulfate were similarly tested. The results and also solubility data are tabulated in Table IV where it will be noted that the fatty acid salts of streptomycin are all of the same order of toxicity, approximately 1700–2000 $\mu\text{g}/20\text{-gm. mouse}$, in contrast to 3350 $\mu\text{g}/20\text{-gm. mouse}$ for the calcium chloride complex salt. Similar figures (approximately 2000 $\mu\text{g}/20\text{-gm. mouse}$) were obtained for the caprylate and undecylenate salts of dihydrostreptomycin, while the toxicity of propionate salt of dihydrostreptomycin was of the same order of toxicity as dihydrostreptomycin sulfate.

DISCUSSION

It is apparent from the present study that the bacterial spectra of the fatty acid salts of streptomycin and dihydrostreptomycin are approximately the same as those for the presently available commercial salts of these drugs. The genera of those organisms susceptible to the commercially available salts were found to be equally susceptible to the fatty acid salts of streptomycin and dihydrostreptomycin, respectively. The individual species of bacteria which were found to have a low resistance to the commercially available streptomycin were found to have a similar low resistance to the fatty acid salts of this drug. Those found to have a high resistance to commercially available streptomycin (*e.g.* *Salmonella*) were found to have a

TABLE IV

Acute intravenous toxicity of various salts of streptomycin and dihydrostreptomycin

Streptomycin salt	LD50 μg/20 gm mouse	Solubility in H ₂ O
Calcium chloride complex	3350	mg/ml Very soluble
Caprylate	1720	8.8
Undecyl-nate*	1860	2.5
Propionate	2025	Very soluble
Dihydrostreptomycin salt		
Sulfate	2700	Very soluble
Caprylate	1920	8.8
Undecyl-nate†	2010	2.5
Propionate	2600	Very soluble

* Salt used as diluent.

† Tested at 2 ml. injected in saline solution.

similar resistance to the fatty acid salts of streptomycin. A similar pattern was demonstrated with dihydrostreptomycin sulfate and the fatty acid salts of dihydrostreptomycin.

When attempts were made to demonstrate the increase in resistance of a strain of *S. typhosa* to the fatty acid salts of streptomycin and dihydrostreptomycin, using as controls the calcium chloride complex salt of streptomycin and dihydrostreptomycin sulfate, it was quite obvious that this organism developed resistance to a considerably less degree to the fatty acid salts than to the commercially available salts of streptomycin and dihydrostreptomycin.

Studies of resistant strains of *S. typhosa* developed through growth in increasing concentrations of the various salts of streptomycin indicate that the resistance developed is specific to a certain degree. A strain of *S. typhosa* made resistant to 5000 $\mu\text{g/ml}$ of the calcium chloride complex salt of streptomycin, although having the same resistance to streptomycin hydrochloride, had only one-eighth as much resistance to streptomycin sulfate, less than one-fiftieth the resistance to the caprylate and undecylenate salts of streptomycin, and approximately one-twenty-fifth the resistance to the propionate salt. Twenty-two transfers of *S. typhosa* in increasing concentrations of the undecylenate, caprylate, and propionate salts of streptomycin resulted in the development of resistance of 30 $\mu\text{g/ml}$, 80 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$, respectively, indicating that the resistance of *S. typhosa* to these salts develops with some difficulty as compared to the resistance developed by this organism when grown in increasing concentrations of the calcium chloride complex salt of streptomycin. It should be pointed out also that when *S. typhosa* is made resistant to the undecylenate and caprylate salts of streptomycin, the resistance developed concomitantly to the streptomycin salts commercially available is considerably greater. Thus, a strain of *S. typhosa* made resistant to 30 $\mu\text{g/ml}$ of the undecylenate salt of streptomycin was found to be resistant to from 312 to 625 $\mu\text{g/ml}$ of streptomycin sulfate, streptomycin hydrochloride, and the calcium chloride complex salt of streptomycin. Since in these studies an intensive effort was made to increase the resistance of a strain of *S. typhosa* to the various streptomycin

salts utilized, it is quite possible that under ordinary conditions little or no resistance would develop to the fatty acid salts. The results of these studies indicate the preparation of new streptomycin and dihydrostreptomycin compounds might well lead to the finding of drugs with improved usefulness. The fatty acid salts are being tested for their effect on experimental infections in animals and for their inhibitory action against tubercle bacilli isolated from cases treated with commercially available salts of streptomycin and dihydrostreptomycin.

It is apparent from the acute toxicity studies of the fatty acid salts of streptomycin and dihydrostreptomycin that the fatty acid salts of streptomycin have approximately twice the acute toxicity of the calcium chloride complex salt of streptomycin, while the propionate salt of dihydrostreptomycin has approximately the same toxicity as dihydrostreptomycin sulfate, and the caprylate and undecylenate salts of dihydrostreptomycin have only a slightly greater toxicity than dihydrostreptomycin sulfate. It should be pointed out that some difficulty was encountered in determining the acute intravenous toxicity because of the insolubility of the caprylate and undecylenate salts of both streptomycin and dihydrostreptomycin.

SUMMARY AND CONCLUSIONS

1. The bacterial spectra of the fatty acid salts of streptomycin and dihydrostreptomycin are approximately the same as that of the streptomycin and dihydrostreptomycin salts commercially available.

2. *S. typhosa* develops resistance to the fatty acid salts of streptomycin and dihydrostreptomycin at a slower rate and to a considerably less degree than to the commercially available salts of these drugs.

3. When *S. typhosa* is made resistant to high concentrations of the calcium chloride complex salt of streptomycin, this organism has relatively low resistance to the fatty acid salts of streptomycin, and, conversely, when *S. typhosa* is made resistant to relatively low concentrations of the fatty acid salts of streptomycin, this organism has a relatively high resistance to the commercially available salts of streptomycin.

4. The anionic portion of the streptomycin and dihydrostreptomycin molecules markedly affects the ability of an organism to develop resistance to these drugs.

5. The fatty acid salts of streptomycin studied are from 1.5 to 2.0 times as toxic as the calcium chloride complex salt of streptomycin when tested intravenously in mice, while the fatty acid salts of dihydrostreptomycin are of approximately the same order of toxicity as dihydrostreptomycin sulfate.

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THE EFFECTS OF NORMAL HORSE SERUM ON THE *IN VITRO* ACTIVITY OF TYROTHRIN¹

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Tyrothricin, discovered by Dubos, is an antibiotic product of *Bacillus brevis*. Its antibacterial action against a wide variety of Gram-positive organisms can be demonstrated by both *in vitro* and *in vivo* tests. As a therapeutic agent, it has been restricted to topical use because of its inherent toxicity on parenteral administration.

Tyrothricin is composed of two fractions, gramicidin and tyrocidine. Except in relatively large amounts tyrocidine has little or no activity against most of the Gram-positive bacteria. On the other hand, gramicidin in very minute amounts exerts a marked antibacterial activity against many of these organisms, and is therefore largely responsible for the major activity of tyrothricin.

As tyrothricin is used in the form of ointments, emulsions, troches, etc. for topical therapy, and as there is some controversy in the literature concerning the effect of serum on the bacteriostatic efficacy of this antibiotic, it seemed desirable to investigate the extent of its reduced activity, if any, in the presence of serum. Dubos (1) stated that the effect of gramicidin is inhibited "only to a small extent by serum, tissue exudates or peptones." Rammelkamp (2), however, noted that serum, exudates and feces markedly inhibit the activity of tyrothricin, and that approximately 100 times the amount of this antibiotic was required to exert an anti-streptococcal activity in their presence. Hotchkiss (3) reported that gramicidin is mildly inhibited by the presence of serum, but that tyrocidine possesses little activity in its presence. Henderson (4) noted that gramicidin, in contrast to tyrocidine, is not affected by soluble proteins, such as serum albumin, "in any concentration," in fact "its activity seems enhanced by serum albumin," a statement he attributes to Dubos.

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METHODS

In our tests, two organisms and three media were employed. The organisms were *Streptococcus fecalis* (M-19) and *Micrococcus pyogenes* var. *aureus* (F.D.A. No. 209). The fecal streptococcus was selected for its high degree of sensitivity to this antibiotic, and *S. aureus* for its prevalence in skin lesions. Three broth media chosen for their different nutritive qualities were used: Edamin (0.8%), Bacto Peptone (1.0%), and Bacto Brain Heart Infusion (3.7%). Cultures in these media incubated at 37° C. were transferred daily, and 1% suspensions utilized in the assay inoculum. The method used for testing the sensitivity of the organisms to tyrothricin was a decimal dilution technic² where varying amounts of an alcoholic solution of tyrothricin were brought into direct contact with the organisms suspended in the assay broths. Bacteriostatic endpoints were determined by visual inspection of the tubes for presence or absence of growth. The tyrothricin used was the accepted standard composed of a mixture of 80% crystalline tyrocidine hydrochloride and 20% crystalline gramicidin in a concentration of 5 mgms. per ml., dissolved in 95% ethyl alcohol. In the assay, the volume of alcohol never exceeded 0.2 ml. per 5 ml. of broth to eliminate the possibility of alcohol affecting the growth of the organisms. All titrations were made in triplicate. In certain instances, where high concentrations of tyrothricin were employed with the fecal streptococcus in the presence of 5% serum, the endpoints could not be determined with any degree of accuracy because of the presence of a precipitate. In such cases the endpoint was determined by the use of a brom thymol blue indicator, growth being indicated by an alkaline reaction. When it became necessary to determine bacteriostasis with the staphylococcus under the same conditions, a nitrate reduction method (5) was employed, growth being indicated by a positive color reaction for nitrite.

RESULTS

Figure 1 illustrates the minimal inhibitory concentrations of tyrothricin required for the two organisms in the three media with and without serum. It can be noted that in the absence of serum the amounts of tyrothricin necessary to cause complete inhibition of *S. fecalis* were 0.01 microgram when Edamin was employed, and 0.1 microgram when both Bacto Peptone and Bacto

² This information will be published in the near future.

INFLUENCE OF SERUM ON THE BACTERIOSTATIC
ENDPOINTS OF TYROTHRICIN

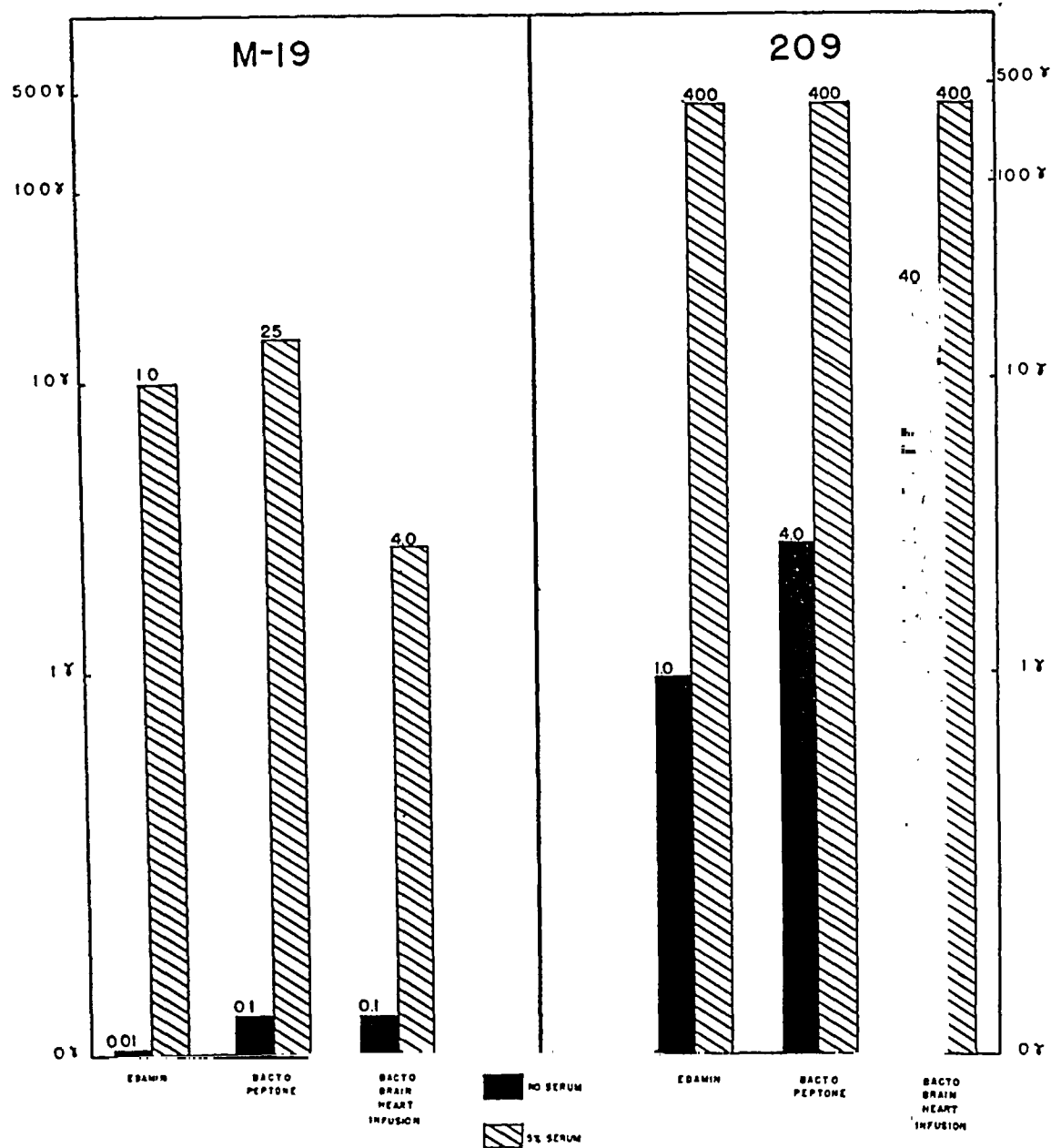


FIG. 1. INFLUENCE OF SERUM ON THE BACTERIOSTATIC ENDPOINTS OF TYROTHRICIN

Brain Heart Infusion broths were used. Upon the addition of 5% normal horse serum to the Edamin broth, it was necessary to use 1,000 times (10.0 micrograms) as much tyrothricin to produce the same degree of bacteriostasis. When serum was added to Bacto Peptone, the amount of tyrothricin necessary to produce the same degree of bacteriostasis was increased 250 fold (25.0 micrograms). A 40-fold increase in the amount of tyrothricin was required to produce bacteriostasis in the presence of serum with the Bacto Brain Heart Infusion broth (4.0 micrograms). As will

be noted in Figure 1, the staphylococcus proved to be approximately 150 times more resistant to the action of tyrothricin than the fecal streptococcus. In spite of this, however, the addition of serum markedly increased the amount of tyrothricin necessary to produce bacteriostasis. Both crystalline gramicidin and crystalline tyrocidine have been tested for their bacteriostatic activity in Edamin broth with and without 5% serum. The streptococcus was inhibited by 20.0 micrograms of tyrocidine in the absence of serum while 200 micrograms were required when serum

was present. On testing the gramicidin fraction with this organism 0.002 microgram was found to be necessary to achieve bacteriostasis in the absence of serum. In its presence, the streptococcus required 2.0 micrograms. Unusual results were obtained when the staphylococcus was tested with tyrocidine. It was found that this fraction was not inhibited by the presence of serum at a concentration of 300 micrograms or more. This amount of tyrocidine was the minimum amount necessary to produce bacteriostasis in the presence and absence of serum. A similar effect was not noted for the gramicidin fraction, however, as 0.2 microgram was required in the absence of serum while 80 micrograms were necessary in its presence.

DISCUSSION

In demonstrating the sensitivity of these organisms to the components of tyrothricin (gramicidin and tyrocidine) it was found that in Edamin broth the fecal streptococcus exhibited a susceptibility to 0.002 microgram of crystalline gramicidin. Its growth was unaffected by 15.0 micrograms of crystalline tyrocidine in this medium. The staphylococcus showed a sensitivity to 0.2 microgram of crystalline gramicidin yet resisted 300 micrograms of crystalline tyrocidine. Because of the vast differences in sensitivity of these organisms to the two components of tyrothricin, any effect of serum on this antibiotic can only be demonstrated by its action on the gramicidin fraction. Although the loss of activity of tyrothricin is attributed to the interference with the gramicidin fraction, there is some indication, however, that when large concentrations (400 micrograms or more) of tyrothricin are present, the tyrocidine fraction may play an important part in antibacterial activity. Although, contrary to reports, we have shown the activity of gramicidin to be greatly reduced by

as little as 5% serum, it cannot be definitely stated that this is entirely due to the direct effect of serum on the gramicidin. From the figures obtained there is some evidence that the serum possibly supplied an added growth factor that might have played a part. Whatever the reason, it is a fact that serum does greatly reduce the activity of tyrothricin tested *in vitro*. It may well be inferred, therefore, that in the presence of undiluted serum exuding from a trauma, where only a thin film of applied tyrothricin is permitted to come into direct contact, its antibiotic activity may be reduced to a point of minor therapeutic value.

SUMMARY AND CONCLUSIONS

It has been shown that the addition of 5% normal horse serum markedly reduced the activity of tyrothricin. The amount of tyrothricin necessary to inhibit the test organisms was markedly influenced by the composition of the medium. From the data obtained it can be concluded that the effective component of tyrothricin is gramicidin. Contrary to published reports, we have found that in inhibitory concentrations tyrocidine is not affected by serum when the test organism is *Staphylococcus aureus*.

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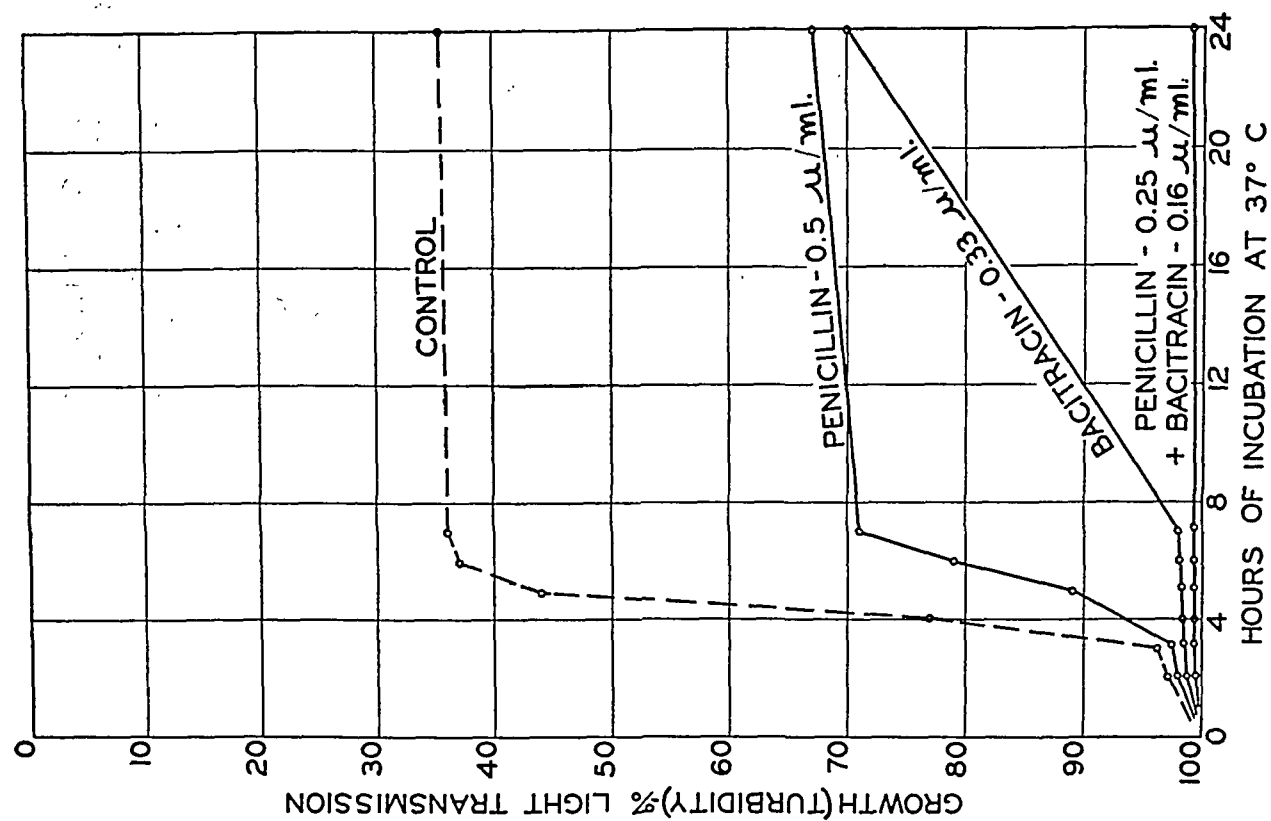


FIG. 2. CULTURE—*Streptococcus* sp. (VIRIDANS GROUP). MEDIUM—TRYPTOSE BROTH—pH 7.3

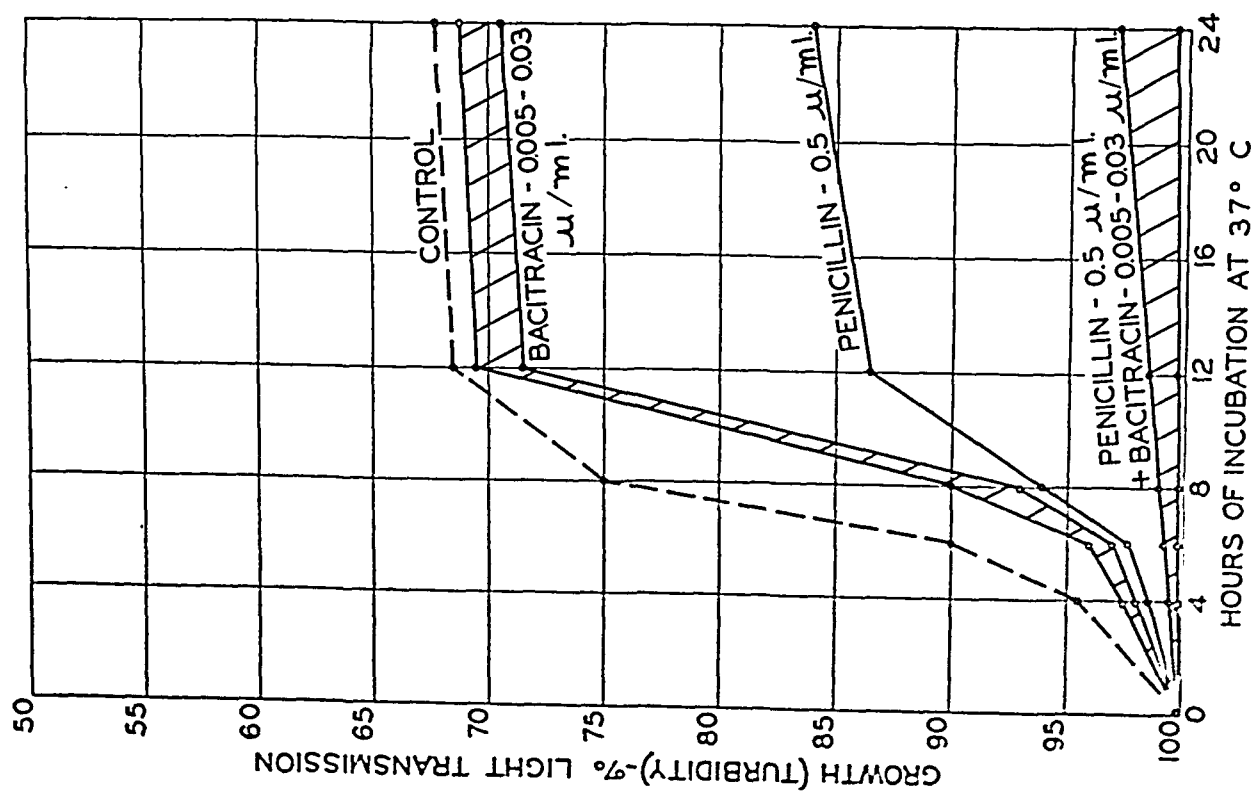


FIG. 1. CULTURE—*Streptococcus faecalis* (160.12). MEDIUM—TRYPTOSE BROTH—pH 7.3

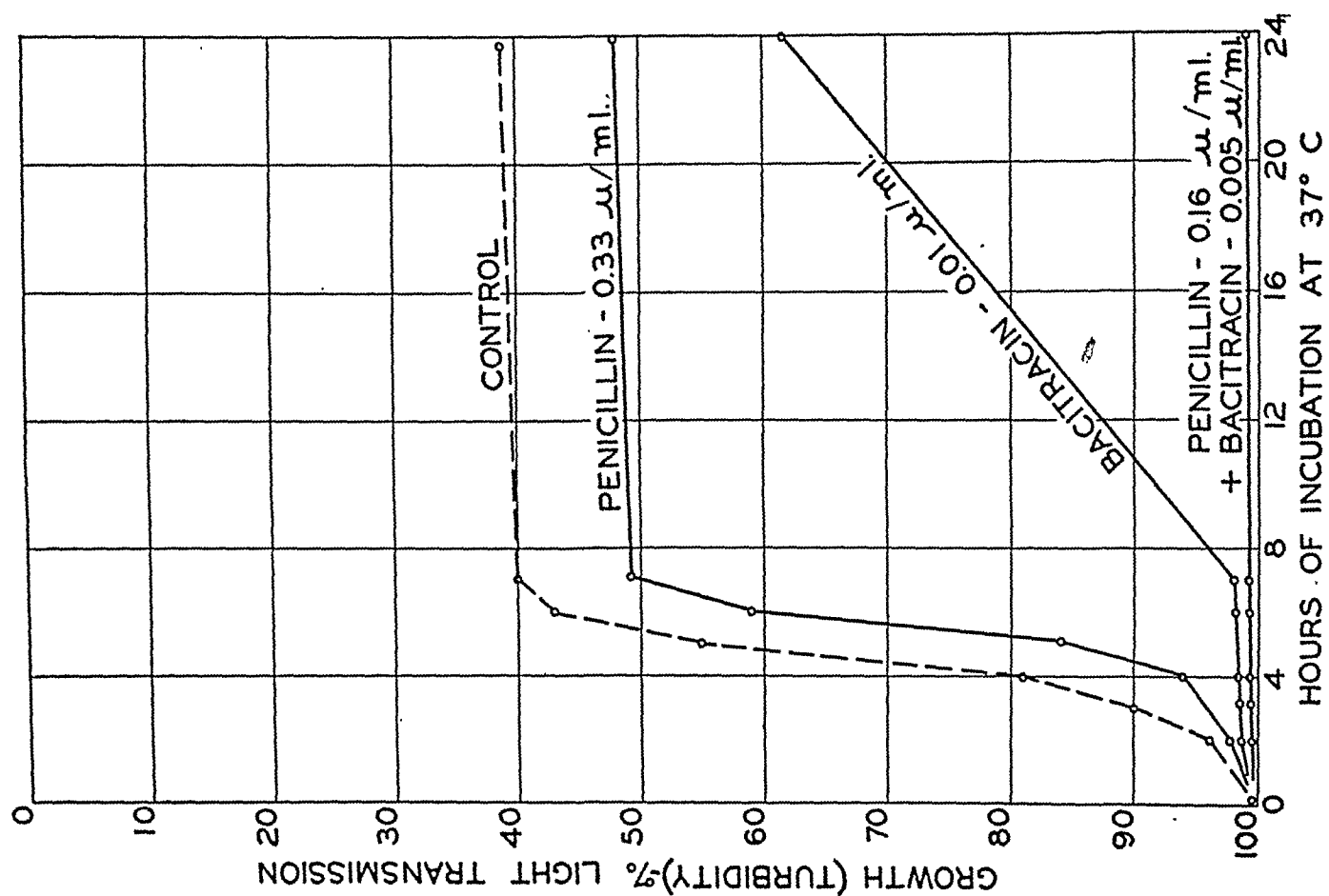


FIG. 4. CULTURE—*Streptococcus* sp. (VIRIDANS GROUP). MEDIUM—TRYPTOSE BROTH—pH 7.2

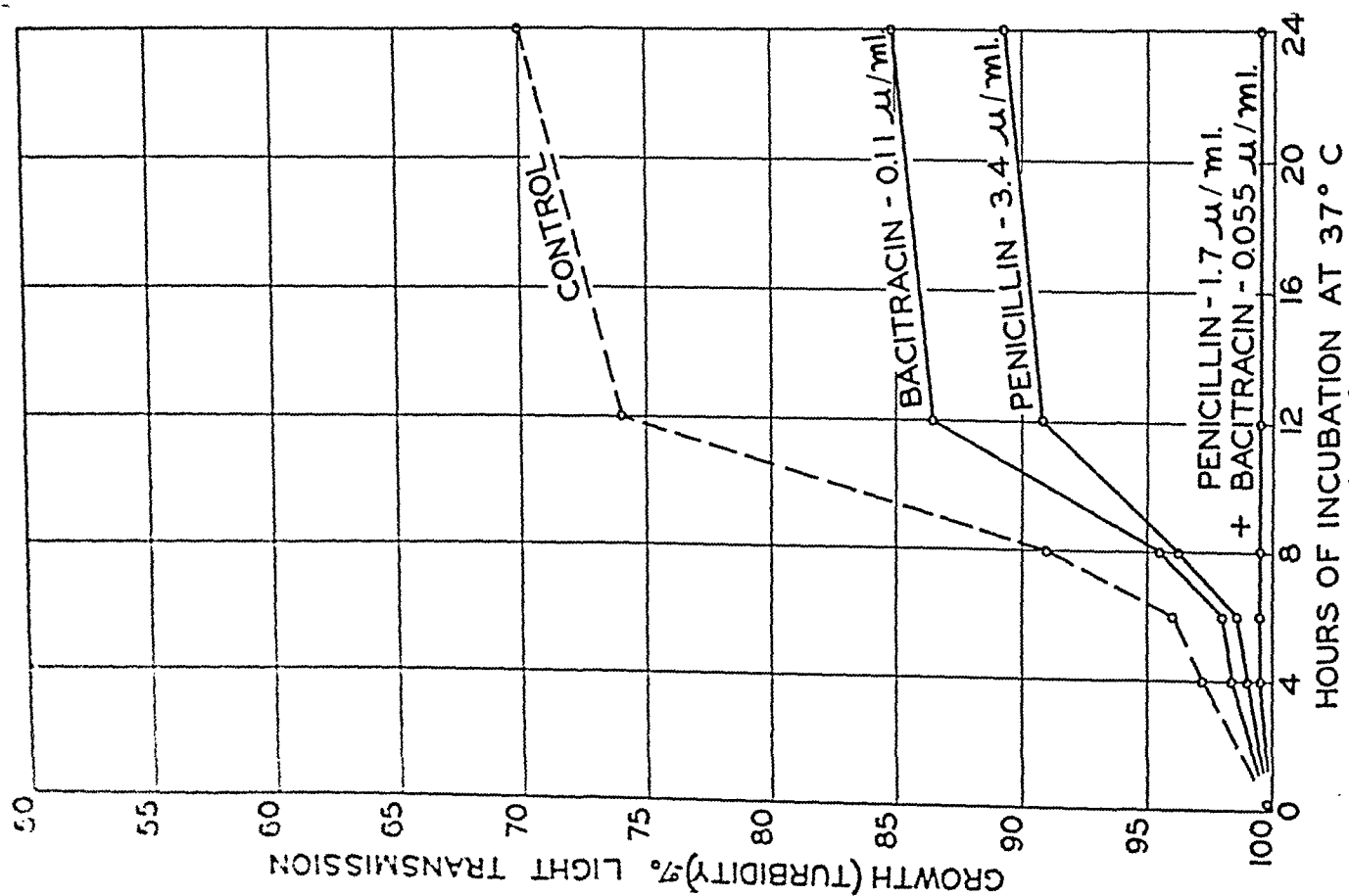


FIG. 3. CULTURE—*Streptococcus* sp. (NON-HEMOLYTIC). MEDIUM—TRYPTOSE BROTH—pH 7.2

COMPARISON OF THE ACTION OF STREPTOMYCIN, POLYMYXIN B, AUREOMYCIN AND CHLOROMYCETIN ON *H. PERTUSSIS*, *H. PARAPERTUSSIS*, *H. INFLUENZAE* AND FIVE ENTERIC STRAINS OF GRAM-NEGATIVE BACILLI^{1, 2}

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Our recent investigations (1) suggest that knowledge of two factors operating *in vitro*—speed of lethal action on sensitive cells and frequency of resistant mutants—offers a reliable basis for predicting the therapeutic efficacy of streptomycin against eight different species of gram-negative bacilli. The data are summarized in Table I. The organisms responsible for infections which have been successfully treated with streptomycin—*H. influenzae*, *E. coli* and *H. pertussis*—have a low incidence of mutants which resist the action of 25 mcg. (micrograms) and 100 mcg. of streptomycin per c.c. In addition, their sensitive cells are killed rapidly by concentrations of streptomycin safely maintained in body fluids (10 mcg. per c.c.). On the other hand, in those infections in which streptomycin has not been found effective therapeutically a concentration of 10 mcg. per c.c. is not rapidly bactericidal for the sensitive cells, and the frequency of mutants resistant to 25 mcg. of streptomycin per c.c. is relatively high—10,000 to 100,000 times the rate of comparable mutants in successfully treated infections (Table I). In *Shigella* and *H. paraptussis* infections, in which streptomycin has not been clinically evaluated, one would predict success in the former and failure in the latter.

In an effort to improve therapeutic results in those infections in which streptomycin has failed therapeutically, either uniformly or as a result of emergence of resistance, three newer antibiotics—polymyxin B, aureomycin and chloromycetin—have been compared with streptomycin in their

action on the same eight species of gram-negative bacilli.

For this purpose three features of their activity have been studied:

1. The minimal concentrations of each agent needed to prevent growth completely over a 48-hour incubation period. A 24-hour period was used for aureomycin for most organisms.

2. The nature of their action on the eight species—primarily bacteriostatic or bactericidal—and the speed of any lethal action.

3. Predictable limitation of their therapeutic efficacy as a result of emergence of resistance and their influence on streptomycin-resistant mutants.

I. Minimal effective concentrations

A simple *in vitro* test, which provided valuable information of streptomycin action on these species, has been used to study the influence of polymyxin B, aureomycin and chloromycetin. This procedure determined the lowest concentration which completely prevented the growth of an inoculum of one to ten million organisms on an optimal agar medium after 48 hours' incubation. Because of the alleged lability of aureomycin *in vitro* the minimal effective concentration of this agent was assessed at 24 hours.

The results are seen in Table II. It is apparent that those organisms which require more than 25 mcg. of streptomycin per c.c. to prevent completely growth under the circumstances described are the causes of infections which have not been treated successfully with streptomycin. Polymyxin B appears to be the most efficient of the four agents and the broadest in its action. Aureomycin and chloromycetin are about equal in their range of action; the growth of each of the eight bacterial species is completely prevented by less than 25 mcg. per c.c. save for *Ps. aeruginosa*; the majority exhibited a high degree of sensitivity.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² The work reported in this communication was supported by grants from The Commonwealth Fund and The National Institutes of Health.

TABLE I
Relation of Frequency of Streptomycin Resistant Mutants and
Speed of Lethal Action of Streptomycin on Sensitive Cells.

Species	Frequency of Mutants Resistant to Concentrations of Streptomycin			Speed of Lethal Action	
	mcg. per c.c.				
	25 mcg. per c.c.	100 mcg. per c.c.	1000 mcg. per c.c.	10 mcg. per c.c.	100 mcg. per c.c.
<i>H. influenzae</i>	10-11	10-11	10-11	2-6 hrs.	10 min.
<i>H. pertussis</i>	10-10 to -11	10-10 to -11	10-10 to -11	4-24 hrs.	2 hrs.
<i>E. coli</i>	10-10	10-10	10-10	6 hrs.	1 hr.
<i>Shigellae</i>	10-9 to -10	10-9 to -10	10-10	4 hrs.	1-2 hrs.
<i>Salmonellae</i>	10-6 to -7	10-9	10-10 to -11	0*	2-4 hrs.
<i>S. typhosa</i>	10-5 to -6	10-8	10-10 to -11	0	4-24 hrs.
<i>H. paraptussis</i>	10-6	10-8	10-10 to -11	0	>24 hrs.
<i>Ps. aeruginosa</i>	10-5 to -6	10-7 to -8	10-10	0	4 hrs

* - Not primarily bactericidal

II. Speed of lethal action

Polymyxin B, aureomycin and chloromycetin have been compared with streptomycin in the speed of their lethal action on small populations (approximately 10,000 to 30,000 organisms per c.c.) in environments which will permit growth. After exposure to the antibiotics for two, six and

24 hours, samples of the broth cultures were examined for the number of viable organisms per c.c. by seeding into appropriate media in pour plate preparations after removal of any inhibitory concentration of the antibiotic, either through dilution or centrifuging and resuspending in normal broth. Table III presents the results on each of the eight species. The per cent of viable organisms remaining after exposure for six hours to two different concentrations of each of the antibiotics is listed.

It is apparent that streptomycin is primarily bactericidal in 10 mcg. per c.c. only against species for which it has been shown to be therapeutically effective. Polymyxin B exerts a rapid lethal action in concentrations of both 1 mcg. and 10 mcg. per c.c. Cultures of all species save for 0.07 per cent in *Salmonella* were sterile after two hours; shorter exposure times have not been studied. Chloromycetin in 10 mcg. per c.c. exerts a more rapid bactericidal action against *H. pertussis* and *H. paraptussis* than does aureomycin; against *H. influenzae* and *S. typhosa* the two agents are equal in the speed of their lethal action. Both aureomycin and chloromycetin are bacteriostatic for all of the other species with the exception of *Ps. aeruginosa*; in a concentration of 100 mcg. per c.c. aureomycin exerts a greater lethal action than does chloromycetin. On the other hand the

TABLE II

MINIMAL EFFECTIVE CONCENTRATIONS OF STREPTOMYCIN (SM), POLYMYXIN B (PMB), CHLOROMYCETIN (CM), AND AUREOMYCIN (AM) FOR 8 SPECIES OF GRAM NEGATIVE BACILLI				
SPECIES	ANTIBIOTIC —mcg./cc.			
	SM	PMB	CM	AM*
<i>H. INFLUENZAE</i>	1-3	0.3	1	0.5-1
<i>H. PERTUSSIS</i>	3	1-5	1	3
<i>E. COLI</i>	75-25	0.5-3	5-<25	5-<25
<i>SHIGELLAE</i>	25	1	3-10	3-5
<i>SALMONELLAE</i>	.25-200	1-5	5	5-<25
<i>S. TYPHOSA</i>	25-75	0.5	3-5	3
<i>Ps. AERUGINOSA</i>	100-200	1-3	>100	50
<i>H. PARAPTUSSIS</i>	>25	5	3	5->10

* 24 hour studies carried for *H. pertussis* and *H. paraptussis*.

results of exposure for 24 hours, not listed here, show that stasis of growth by chloromycetin persists through 24 hours in cultures of all of these organisms save for *Ps. aeruginosa*, and the number of viable cells even decreases significantly, whereas the static effect of aureomycin is seen after 24 hours only against *Shigella* and *S. typhosa*; these results may only reflect the deterioration of aureomycin. However the persistence of bacteriostasis with some organisms suggests that other factors are playing a role.

Ps. aeruginosa is not even inhibited by a six-hour exposure to streptomycin, chloromycetin or aureomycin in a concentration of 10 mcg. per c.c., whereas polymyxin B in a concentration of 1 mcg. per c.c. is bactericidal within two hours.

The greater bactericidal action of 100 mcg. per c.c. of streptomycin is worthy of emphasis since in infections of the meninges and urinary tract a comparable concentration can be maintained in these fluids without damage in most patients.

III. Resistance problem

The study of the limitation of therapeutic efficacy of these agents by the phenomenon of resistance was approached in two ways: 1) examination of large populations for presence of cells resistant to these antibiotics, and 2) examination of

TABLE III

PER CENT VIABLE CELLS AFTER 6 HRS. EXPOSURE TO STREPTOMYCIN (SM), POLYMYXIN B (PMB), CHLOROMYCETIN (CM), AND AUREOMYCIN (AM).								
SPECIES	SM		PMB		CM		AM	
	mcg./cc.		mcg./cc.		mcg./cc.		mcg./cc.	
	10	100	1	10	10	100	10	100
H. INFLUENZAE	0	0	0	0	0	0	0.96	0
H. PERTUSSIS	0.03	0	0	0	0.1 2.0	0.03 3.0	10	0.1
E. COLI	0	0	0	0	100	52 55	128	1.8
SHIGELLAE	0	0	0	0	100	65 79	100	20
SALMONELLAE	1.4 100	0	0.01	0	100	26 35	100	8.6
S. TYPHOSA	37 633	0 0.4	0	0	54 60	12 22	74	0.2
PS. AERUGINOSA	287 1500	0	0	0	10,100	15	170	2.4
H. PARAPERTUSSIS	100	10 25	0	0	0.4 0.5	1.0	2 23	0 0.1

TABLE IV

MINIMAL EFFECTIVE CONCENTRATIONS OF POLYMYXIN-B (PMB), CHLOROMYCETIN (CM), AND AUREOMYCIN (AM) FOR STREPTOMYCIN (SM) RESISTANT MUTANTS OF 8 SPECIES.				
SPECIES	ANTIBIOTIC - mcg./cc.			
	SM	PMB	CM	AM
H. INFLUENZAE	>1000	0.1	1	0.5
H. PERTUSSIS	>1000	5	1	0.5
E. COLI	>1000	0.3-3	5-<25	3-5
SHIGELLAE	>1000	0.5	1	3-5
SALMONELLAE	>1000	1-3	5	3-5
S. TYPHOSA	>1000	0.3-0.5	3	3
PS. AERUGINOSA	>100	1-3	25->100	3-<25
H. PARAPERTUSSIS	>1000	5-25	1-3	1-3

E. SHIMAKOV

their action on streptomycin-resistant mutants of each of these species.

It has already been reported (2-4) that mutants resistant to 1000 mcg. of streptomycin per c.c. may be easily demonstrated in large populations of each of the eight species; mutants resistant to 25 mcg. and 100 mcg. of streptomycin per c.c. are uniformly present in smaller populations of the species against which this antibiotic has not been effective therapeutically (1).

Large populations, 60 to 600 billions, of each of the eight species have been examined for presence of cells resistant to polymyxin B, 10 mcg. and 100 mcg. per c.c. and chloromycetin 10 mcg. per c.c.; the supply has not permitted study of a higher concentration. *H. pertussis* and *H. parapertussis* have been examined for cells resistant to 10 mcg. and 100 mcg. of aureomycin per c.c.

It was not possible to demonstrate cells resistant to 100 mcg. per c.c. of polymyxin B in any species by this method, and only in *Salmonella* and *Ps. aeruginosa* was it possible to demonstrate a minute fraction of cells resistant to 10 mcg. per c.c.; when a few of the colonies of these two species which formed in agar containing 10 mcg. per c.c. were tested for their sensitivity to this concentration only an occasional colony grew in polymyxin B agar (10 mcg. per c.c.) but growth was normal on control medium.

While a few colonies grew when large populations of *E. coli*, *Salmonella* and *S. typhosa* were seeded in pour plate preparations of agar containing 10 mcg. per c.c. of chloromycetin, none of them on subculture grew on chloromycetin agar (10 mcg. per c.c.).

The investigation of resistance of these newer agents is still in progress but it is already apparent that resistance to polymyxin B and chloromycetin differs from streptomycin resistance; the data suggest that emergence of resistance will play a less important role in therapy.

The influence of polymyxin B, chloromycetin and aureomycin on cells highly resistant to streptomycin is shown in Table IV for each of the eight species. The sensitivity of these streptomycin-resistant strains does not differ significantly from those strains which are sensitive to streptomycin. Therefore it is apparent that the mechanism of action of each of these agents differs from the mode of action of streptomycin.

These data suggest a number of clinical applications. Polymyxin B would be the treatment of choice for all of these infections, but its toxic action precludes its clinical use unless the injurious substance can be removed without altering the antibacterial action. A final assessment of aureomycin *in vitro* must await additional factual data. Chloromycetin because of absence of toxic manifestations, its rapid lethal action on *H. influenzae* and *H. pertussis* and the rarity of resistant mutants warrants a clinical trial in these infections. In *E. coli* and *Shigella* infections there

is reason to anticipate a more rapid elimination of the organisms by the combined action of sulfadiazine and streptomycin than by aureomycin or chloromycetin alone. In *Salmonella* and *S. typhosa* infections, either chloromycetin or aureomycin should be a more effective agent than streptomycin. For *Ps. aeruginosa* infections we can expect little therapeutic value from streptomycin, aureomycin or chloromycetin used as a single agent if concentrations in the body fluids do not exceed 10 mcg. per c.c. The results suggest that the combined action of streptomycin and aureomycin may prove of therapeutic value in *Ps. aeruginosa* infections of body fluids in which concentrations of 50 mcg. to 100 mcg. per c.c. can be maintained with safety. In *H. paraptussis* infections chloromycetin should prove to be the treatment of choice.

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EXPERIMENTAL STUDIES ON THE ACTION OF STREPTOMYCIN, AUREOMYCIN, AND CHLOROMYCETIN ON BRUCELLA^{1,2}

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The use of streptomycin in the treatment of brucellosis revealed that most of the patients failed to respond satisfactorily to its administration, and among those who did respond, relapses almost always followed (1). It was subsequently observed that when sulfadiazine was given simultaneously with streptomycin, the result was much more encouraging (2-4). However, this combination was also followed by relapses (5). *Brucella* could be recovered after treatment from the blood of approximately 25 per cent of the patients with infections due to *Br. abortus* and 45 per cent of those with disease due to *Br. melitensis*.

In this clinic the failure of streptomycin and sulfadiazine to eradicate *Brucella* consistently from the tissues prompted the study of why such a therapeutic combination failed, and stimulated the search for more effective antibrucella agents. The problem was approached by *in vitro* studies, animal experiments, and, finally, by the clinical trial of newer antibiotic agents. This report is concerned primarily with the results of the *in vitro* experiments.

I. STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

In vitro Sensitivity of *Brucella*

Forty-one strains of *Brucella* recently isolated from patients were tested for sensitivity to streptomycin and dihydrostreptomycin.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and by a grant from the Lederle Laboratories. The aureomycin was supplied by the Lederle Laboratories; the dihydrostreptomycin, by E. R. Squibb Company; polymyxin, by the Stamford Research Laboratories, American Cyanamid Company; bacitracin and Q-19, by the Upjohn Company; and chloromycetin, by Parke, Davis and Company.

Method: Solutions of streptomycin and dihydrostreptomycin were prepared by adding 10 ml. of physiologic saline solution to 1.0 gram of base of the drugs. The stock solution was stored at 4° C. and was diluted to a concentration of 1000 or 100 µg. per ml. immediately before the sensitivity tests were performed. To each of 12 test tubes (12 × 100 mm.), except the first, was added 0.5 ml. of tryptose phosphate broth. The first two tubes were inoculated with 0.5 ml. each of the test solution of streptomycin or dihydrostreptomycin. Two-fold dilutions of the drug were made by transferring 0.5 ml. of the mixture in the second tube to the third tube, and so on, through the 11th tube. The 12th tube contained only broth and organisms. Each of the 12 tubes was then inoculated with 1.5 ml. of a 1:100 dilution of a 24-hour culture in tryptose phosphate broth. Thus, the first tube contained a 1:4 dilution of the test solution of the antibiotic; the second, 1:8; third, 1:16, through the 11th, which contained a 1:4096 dilution. The cultures of *Br. abortus* were incubated for 48 hours at 37° C. in an environment containing a 10 per cent concentration of carbon dioxide, while the strains of *Br. suis* and *Br. melitensis* were subjected to normal atmospheric conditions. At the end of this time, the tubes were examined for gross turbidity and the contents of each tube streaked on tryptose agar. The sensitivity of the organism was reported as the minimum concentration of streptomycin or dihydrostreptomycin required to kill all the organisms.

Results: Thirteen strains of *Br. abortus*, ten of *Br. melitensis*, and one of *Br. suis*, all isolated before being exposed to streptomycin, were found to be sensitive to concentrations between 1.0 and 2.5 µg. of streptomycin and dihydrostreptomycin per ml. The results are presented in Tables I and II.

The Bactericidal Action of Streptomycin on Brucella

The effect of streptomycin on the multiplication and viability of *Brucella* was studied in the following manner.

Methods: A saline suspension of organisms, equal in turbidity to a barium sulfate No. 1 standard, was prepared from a 48-hour agar culture of *Br. abortus*. Three tubes containing 10 ml. of tryptose phosphate broth were inoculated with 0.05 ml. of the bacterial suspension so that the final concentration of organisms was approximately 5,000,000 bacteria per ml. Sufficient streptomycin was added to each of two tubes to make a concentration of

TABLE I

Sensitivity of Brucella abortus to streptomycin and dihydrostreptomycin

Strain	Streptomycin (μg./ml.)		Dihydrostreptomycin (μg./ml.)	
	Minimum conc. inhibiting growth	Maximum conc. permitting growth	Minimum conc. inhibiting growth	Maximum conc. permitting growth
Lynch	1.2	0.6	1.6	0.8
Kreutz	2.0	1.0	1.2	0.6
Kreutz*	1.2	0.6	1.2	0.6
Rivera	1.2	0.6	1.2	0.6
Rivera*	2.0	1.0	1.2	0.6
Archer*	1.2	0.6		
Archer*	1.2	0.6	1.2	0.6
Steinbrecker	1.2	0.6	1.2	0.6
Steinbrecker*	1.2	0.6	1.2	0.6
Wimmer	1.2	0.6	1.2	0.6
Wimmer*	1.2	0.6		
Garrity	2.0	1.0		
Garrity*	2.0	1.0		
Shaeffer	1.0	0.5	1.0	0.5
Hamilton	2.0	1.0	1.6	0.8
Elsted	1.0	0.5	1.6	0.8
Elsted†	10,000	7,500	10,000	7,500
Guck	1.6	0.8	1.6	0.8
Nelson	1.6	0.8	1.6	0.8
Johnson	1.6	0.8	1.6	0.8
Schweim	1.6	0.8	1.6	0.8

* Strain isolated after streptomycin-sulfadiazine therapy

† Isolated after streptomycin therapy

10 μg. per ml. in one tube and 20 μg. per ml. in the other. The third tube was used as a control. The same procedure was carried out using cultures of *Br. melitensis* and *Br. suis*. The tubes were incubated under the proper tension of carbon dioxide at 37° C., and a standard loopful of the broth from each tube was subcultured on streptomycin-free agar at 15-minute intervals during the first hour, at three and six hours after inoculation, and then daily for 14 days. The subcultures were incubated for 96 hours and examined for growth. The broth cultures were inspected for evidence of growth turbidity at the time of each subculture.

Results: No evidence of bacterial multiplication appeared in the broth cultures containing streptomycin, while in the control tubes there was a turbid growth at the end of the first 24 hours of incubation. No viable *Brucella* could be recovered from the tubes containing streptomycin after three hours of incubation, whereas the control cultures contained living organisms throughout the 14 days of observation (see Tables III and IV).

Resistance of Brucella to the Antibacterial Action of Streptomycin and Dihydrostreptomycin

The rapid development of resistance of gram-negative bacilli to streptomycin has been a frequent

TABLE II

Sensitivity of Brucella melitensis and Brucella suis to streptomycin and dihydrostreptomycin

Strain	Streptomycin (μg./ml.)		Dihydrostreptomycin (μg./ml.)	
	Minimum conc. inhibiting growth	Maximum conc. permitting growth	Minimum conc. inhibiting growth	Maximum conc. permitting growth
<i>Br. melitensis</i>				
3752	2.5	1.2	1.2	0.6
3752*	2.5	1.2	2.5	1.2
3925	2.5	1.2	1.2	0.6
3925*	>50,000	>50,000	>50,000	>50,000
2716	2.5	1.2	1.2	0.6
2716*	2.5	1.2	2.5	1.2
3777	2.5	1.2	2.5	1.2
3777*	2.5	1.2	2.5	1.2
3988	2.5	1.2	1.2	0.6
3988*	2.5	1.2	1.2	0.6
4103	2.5	1.2	1.2	0.6
4103*	2.5	1.2	2.5	1.2
3858	2.5	1.2	1.2	0.6
3858*	2.5	1.2	2.5	1.2
4241	1.5	1.0		
4241†	1.5	1.0	1.2	0.6
4185	1.5	1.0	1.2	0.6
4185†	1.5	1.0		
4290	1.5	1.0		
4290†	1.5	1.0		
<i>Br. suis</i>				
374	1.2	0.6	1.2	0.6

* Isolated after streptomycin-sulfadiazine therapy

† Isolated after aureomycin therapy

cause of the failure of the antibiotic in the treatment of human infections. The most acceptable theory of the origin of streptomycin-resistant strains of bacteria is that they arise from innately resistant organisms normally present in small numbers in every large population of a sensitive strain (6). The presence of naturally occurring streptomycin-resistant organisms in sensitive strains of *Brucella* was demonstrated by exposing

TABLE III

Bactericidal action of streptomycin on Brucella abortus

Time Interval	Control		Streptomycin (10 μg./ml.)		Streptomycin (20 μg./ml.)	
	Broth turbidity	Sub-culture	Broth turbidity	SM-free sub-culture	Broth turbidity	SM-free sub-culture
5 min.	—	++	—	++	—	++
15 min.	—	++	—	++	—	++
30 min.	—	++	—	++	—	++
1 hour	—	++	—	+	—	+
3 hours	—	++	—	—	—	—
6 hours	—	++	—	—	—	—
24 hours	+	+++	—	—	—	—
2 days	+++	++++	—	—	—	—
3 days	++++	++++	—	—	—	—
4 days	++++	++++	—	—	—	—

TABLE IV
Bactericidal action of streptomycin on *Brucella melitensis* and *Brucella suis*

Time interval	<i>Brucella melitensis</i>				<i>Brucella suis</i>			
	Control		Streptomycin (20 µg./ml.)		Control		Streptomycin (20 µg./ml.)	
	Broth turbidity	Subculture	Broth turbidity	SM-free subculture	Broth turbidity	Subculture	Broth turbidity	SM-free subculture
5 min.	—	+++	—	++	—	++	—	++
15 min.	—	+++	—	++	—	++	—	++
30 min.	—	+++	—	++	—	++	—	++
1 hour	—	+++	—	+	—	++	—	+
3 hours	—	+++	—	—	—	++	—	—
6 hours	—	+++	—	—	—	++	—	—
24 hours	++	+++	—	—	++	+++	—	—
2 days	++++	++++	—	—	++++	++++	—	—
3 days	++++	++++	—	—	++++	++++	—	—
4 days	++++	++++	—	—	++++	++++	—	—

large bacterial populations to varying concentrations of streptomycin.

Method: A concentrated suspension of organisms was prepared by heavily inoculating a large tryptose agar

slant with a culture of *Brucella*. After 48 hours of incubation, the bacteria were washed from the slant with 1.0 ml. of tryptose phosphate broth. The suspension of organisms was spread evenly over the agar surface of bottles, prepared by adding 60 ml. of melted agar to 16

Survival of Variants of *Brucella Abortus* Resistant to Streptomycin

524

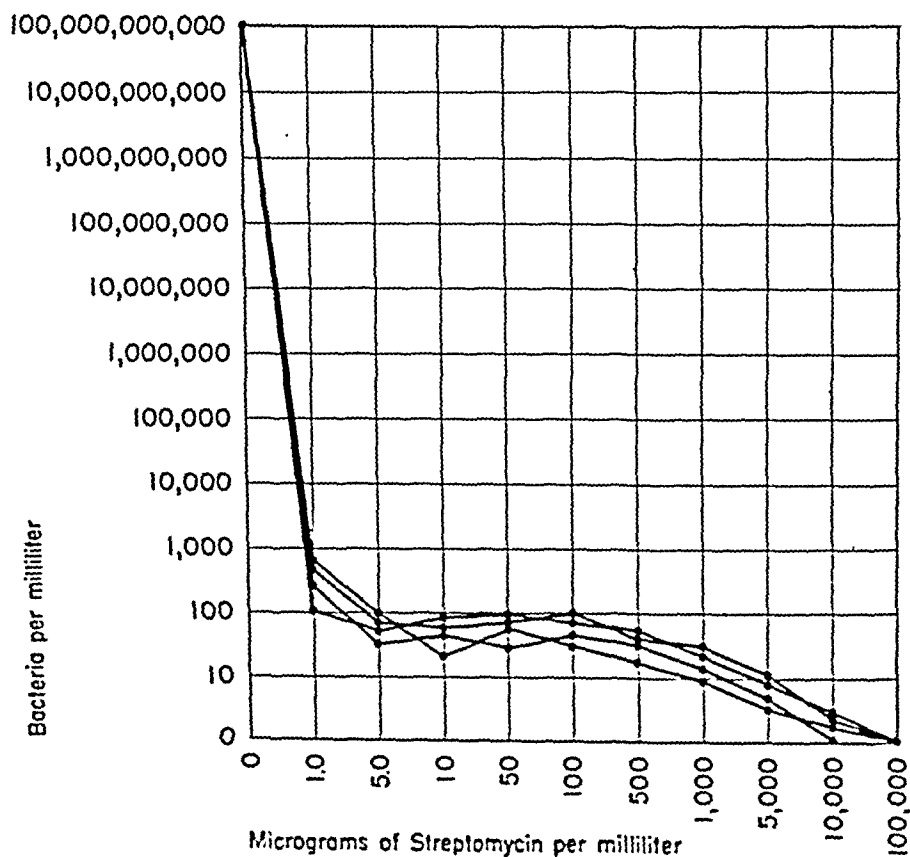


FIG. 1

oz. oval prescription bottles with screw caps. The bottles were incubated with the inoculated agar surface down for 48 hours, and then the bacterial growth was resuspended in 5 ml. of broth. Bacterial counts on the suspension of organisms were performed with a photoelectric colorimeter by comparing the turbidity of a 10^{-3} dilution to a barium sulfate standard No. 1. The concentration of bacteria was adjusted to approximately 100 billion organisms per ml. and then exposed to varying concentrations of streptomycin. Eleven pour plates were prepared, using 1.0 ml. of the bacterial suspension, 9.0 ml. of tryptose agar, and sufficient concentrations of streptomycin to make final concentrations of 1.0, 2.5, 5.0, 10, 50, 100, 500, 1,000, 5,000, 10,000, and 100,000 $\mu\text{g. per ml.}$ Each experiment was performed in triplicate and the plates were incubated in jars at 37°C. Colony counts were performed weekly for a month.

Results: The number of the surviving streptomycin-resistant variants decreased very rapidly as the concentration of streptomycin increased from 1.0 to 5.0 $\mu\text{g. per ml.}$, then more gradually to a concentration of 10,000 $\mu\text{g. per ml.}$, after which

there were no surviving variants. Streptomycin survival curves for *Br. abortus* are shown in Figure 1, and for *Br. suis*, in Figure 2.

Some of the streptomycin-resistant colonies were found to be largely dependent upon streptomycin for growth. The dependency of the colonies upon streptomycin was determined by adding suspensions of the colonies to pour plates with and without the addition of 100 $\mu\text{g.}$ of streptomycin per ml. The resistant colonies grew equally well in the presence or absence of streptomycin, whereas the dependent colonies multiplied only in the plates containing the antibiotic. These dependent strains would grow if streaked heavily on streptomycin-free agar, but not as luxuriously as non-dependent strains. Streptomycin-dependent strains of *Brucella* grew equally well in the presence of dihydrostreptomycin, and strains dependent upon dihydrostreptomycin multiplied in media containing streptomycin. Media containing

Survival of Variants of *Brucella Suis* Resistant to Streptomycin

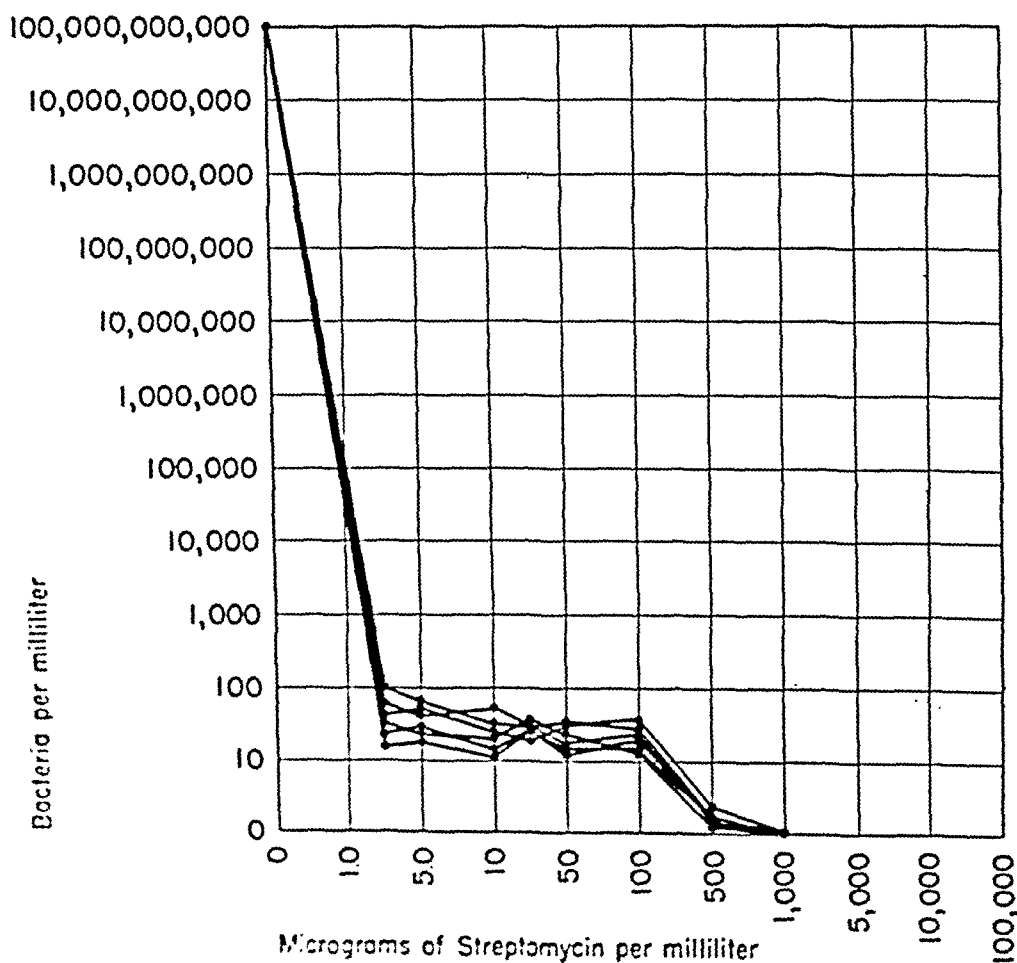


FIG. 2

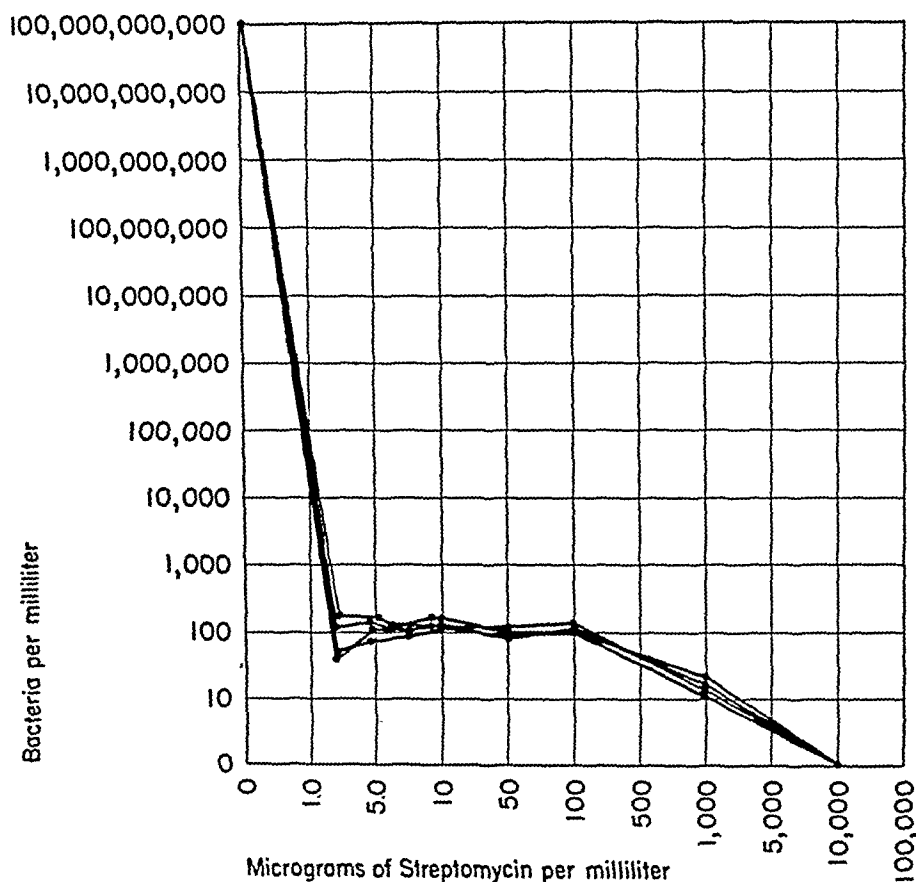
Survival of Variants of *Brucella Abortus* Resistant to Streptomycin
Elsted

FIG. 3

aureomycin or chloromycetin did not support the growth of streptomycin-dependent strains.

Sensitivity of Strains of *Brucella* Isolated after Streptomycin Therapy

Streptomycin resistance as a cause of therapeutic failure in cases of brucellosis was investigated, first, by determining the sensitivity of the strains isolated after therapy as compared to the sensitivity of the strains isolated from the same patient before therapy; and, secondly, by comparing the distribution and number of the resistant variants occurring in these strains of *Brucella*.

Fifteen strains of *Brucella* isolated after streptomycin therapy were tested for sensitivity to streptomycin. Seven strains of *Br. abortus* and six strains of *Br. melitensis* cultured after combined sulfadiazine and streptomycin therapy showed no change in sensitivity to streptomycin. One strain of *Br. abortus* obtained after treatment

with only streptomycin was studied by Hall and Spink (7) and was found to have increased in resistance from 1.0 $\mu\text{g.}$ per ml. to 10,000 $\mu\text{g.}$ per ml. One strain of *Br. melitensis*, isolated after combined streptomycin and sulfadiazine therapy, changed in sensitivity from 2.5 $\mu\text{g.}$ to 50,000 $\mu\text{g.}$ of streptomycin per ml.

The two strains of *Brucella* which developed resistance to streptomycin were studied for evidence of streptomycin dependency. The strain of *Br. melitensis* did not depend upon the presence of streptomycin for growth, but the resistant strain of *Br. abortus* contained predominantly colonies that were smaller than normal and grew on streptomycin-free agar only when a heavy inoculum was used. The growth of the small colonies was greatly enhanced by the addition of from 50 to 1,000 $\mu\text{g.}$ of streptomycin per ml. (7).

The number of the resistant variants present in the strains isolated after therapy was compared

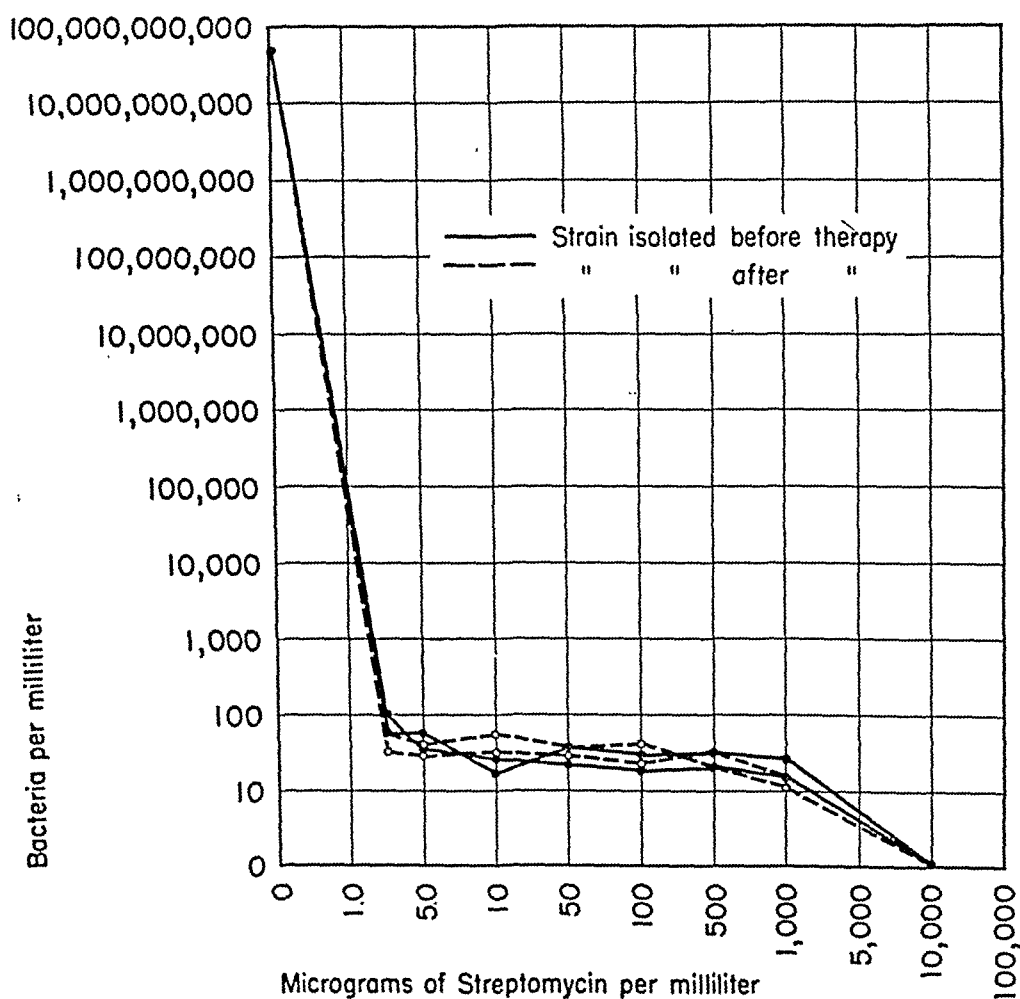
Survival of Variants of *Brucella Abortus* Resistant to Streptomycin

FIG. 4

TABLE V

Survival of variants of *Brucella abortus* in a bacterial population of 100 billion organisms resistant to 100 μ g. per ml. of streptomycin

	Rivera	Kreutz	Steinbrecker	Wimmer	Garrity	Archer	Elsted	Lynch
Strain isolated before therapy	74	52	61	46	56	—	63	52
	96	35	51	57	48		91	63
	84	44	54	60	63		96	48
	31	96	48	73	71		82	59
	70	72	38	82	52		79	79
	81	83	48	53	49		46	82
	91	56	52	75	47		62	49
	42	65	46	41	64		68	67
	68	76	39	87	59		85	63
	72	81	45	69	56		83	85
Strain isolated after therapy	39	95	40	47	45	64 78 62 75 79 68 56 63 84 77	Innumerable	—
	48	77	30	94	51			
	69	75	25	96	67			
	41	58	30	79	45			
	86	91	62	91	51			
	68	62	37	82	62			
	59	73	27	58	55			
	62	85	48	62	49			
	72	64	57	82	57			
	68	59	46	79	60			

TABLE VI

Survival of variants of *Brucella melitensis* in a bacterial population of 100 billion organisms resistant to 100 µg. per ml. of streptomycin

	2716	3988	4103	3925	472
Strains isolated before therapy	240 230 236 239 233	230 228 234 230 241	240 229 230 221 224	280 271 279 286 266	246 200 266 232 244
Strains isolated after therapy	200 263 220 210 208	234 226 220 239 230	269 241 226 230 237	Innum- erable	—

to the number present in strains isolated from the same patients before therapy and to strains isolated from patients who responded satisfactorily to treatment. The streptomycin survival curve of a strain of *Br. abortus* (Elsted) which subsequently became resistant to streptomycin is shown in Figure 3. There is no significant difference between this curve and that of the control strain (Lynch) which responded to therapy (Figure 1), and, as seen in Figure 4, the curve of the surviving resistant variants of a strain isolated after therapy

TABLE VII

Sensitivity of *Brucella abortus* to aureomycin

Strain	Minimum conc. inhibiting growth µg./ml.	Maximum conc. permitting growth µg./ml.	Sterilizing concentration µg./ml.
Lynch	1.0	0.8	125
Kreutz	1.2	0.6	62.5
Kreutz*	1.2	0.6	
Rivera	1.2	0.6	
Rivera*	1.2	0.6	62.5
Archer*	1.2	0.6	
Archer*	1.2	0.6	
Steinbrecker	1.2	0.6	62.5
Steinbrecker*	1.2	0.6	62.5
Wimmer	1.2	0.6	
Wimmer*	1.2	0.6	
Garrity	1.2	0.6	
Garrity*	1.2	0.6	
Shaeffer	1.0	0.5	62.5
Hamilton	1.2	0.6	
Elsted	1.0	0.5	
Elsted†	1.0	0.5	
Guck	1.2	0.5	
Nelson	1.2	0.6	
Johnson	1.2	0.6	
Schweim	1.2	0.6	
Schweim‡	1.2	0.6	

* Isolated after streptomycin-sulfadiazine therapy

† Streptomycin resistant

‡ Isolated after aureomycin therapy

is identical to the curve of the strain cultured from the same patient before therapy.

In order to obtain data on the number of resistant organisms present in a large number of strains of *Brucella*, these strains were exposed to an arbitrary concentration of 100 µg. of streptomycin per ml. These studies revealed that the number of variants present in strains obtained after therapy did not vary significantly from the number of survivors present in strains isolated before therapy. The number of resistant survivors found in the cultures of *Br. melitensis* was greater than the number of survivors present in strains of *Br. abortus* (Tables V and VI).

Other properties of the strains of *Brucella* recovered from patients after the completion of streptomycin therapy were studied. There were no changes in the gross colony or growth characteristics, or were changes in cell morphology noted. There were no differences in the susceptibility of these strains to the bactericidal effect of normal human serum or convalescent brucellosis serum, nor in their sensitivity to other antibiotics.

TABLE VIII

Sensitivity of *Brucella melitensis* and *Brucella suis* to aureomycin

Strain	Minimum conc. inhibiting growth µg./ml.	Maximum conc. permitting growth µg./ml.	Sterilizing concentration µg./ml.
<i>Br. melitensis</i>			
3752	0.8	0.6	62.5
3752*	1.4	1.2	
3925	0.8	0.6	
3925†	0.8	0.6	
2716	0.8	0.6	
2716*	1.4	1.2	
3777	1.4	1.2	
3777*	1.4	1.2	
3988	0.8	0.6	62.5
3988*	1.2	1.0	
4103	0.8	0.6	
4103*	1.4	1.2	125
3858	0.8	0.6	
3858*	0.8	0.6	
4241	1.5	1.0	
4241‡	1.5	1.0	
4185	1.5	1.0	
4185‡	1.5	1.0	
4290	1.5	1.0	
4290‡	1.5	1.0	
<i>Br. suis</i>			
374	1.2	0.6	125

* Isolated after streptomycin-sulfadiazine therapy

† Streptomycin resistant

‡ Isolated after aureomycin therapy

TABLE IX
The action of aureomycin on *Brucella abortus*

Time interval	Control		Aureomycin (1 µg./ml.)		Aureomycin (10 µg./ml.)		Aureomycin (100 µg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	AM-free subculture	Broth turbidity	AM-free subculture	Broth turbidity	AM-free subculture
30 min.	—	++	—	++	—	++	—	++
1 hour	—	++	—	++	—	++	—	+
6 hours	—	++	—	+	—	++	—	+
24 hours	+	+++	—	+	—	+	—	—
2 days	++	++++	+	++	—	+	—	—
3 days	+++	++++	++	+++	—	+	—	—
4 days	++++	++++	+++	++++	+	++	—	—
6 days	++++	++++	++++	++++	++	+++	—	—
10 days	++++	++++	++++	++++	++++	++++	—	—
14 days	++++	++++	++++	++++	++++	++++	—	—

II. AUREOMYCIN

Studies with aureomycin were approached, first, by investigating the *in vitro* activity of the antibiotic against the three species of *Brucella*; secondly, by an evaluation of its action against experimentally produced infections in the chick embryo, mouse, and guinea pig; and finally, by its clinical trial in the treatment of patients with brucellosis (8, 9). The results of the *in vitro* experiments will be summarized in this report.

In vitro Sensitivity

Forty-four strains of *Brucella*, including 23 strains of *Br. abortus*, 20 strains of *Br. melitensis*, and one strain of *Br. suis*, were tested for sensitivity to aureomycin.

Method: The broth dilution method was used and varied slightly from that described for the determination of streptomycin sensitivity. The stock solution of aureomycin was prepared by adding 20 ml. of a sterile saline solution to a vial containing 20 mg. of aureomycin hydrochloride. The stock solution was stored at 4° C. for as long as a month and diluted to a concentration of 100 µg. per ml. the day the sensitivity tests were performed. In contrast to the values expressed for streptomycin and dihydrostreptomycin, the sensitivity of *Brucella* to aureomycin was reported as that concentration of the antibiotic required to prevent the multiplication of the bacteria, as measured by broth turbidity. The concentration actually sterilizing the cultures was determined in some of the tests by subculturing the broth from each tube on agar, and was reported as the minimal concentration of the drug from which no live organisms could be recovered.

Results: The sensitivity of the strains of *Brucella* to aureomycin varied from 0.6 to 1.5 µg. per ml., though concentrations as high as 62.5 to 125 µg. of aureomycin per ml. were required to steri-

lize the cultures (see Tables VII and VIII). Two strains of *Brucella* resistant to streptomycin showed no comparable increased resistance to aureomycin.

The Action of Aureomycin on *Brucella*

The technique of the experiment was identical to that described already for streptomycin, except that the concentrations of aureomycin employed were 1.0, 10, and 100 µg. per ml.

Results: The broth containing 1.0 µg. of aureomycin per ml. became turbid on the second day of incubation, one day later than the control. Broth turbidity developed in the tube containing 10 µg. per ml. on the fourth day, but there was no evidence of multiplication of *Brucella* in the broth containing 100 µg. of aureomycin per ml. Organisms were consistently recovered from the tubes containing 1.0 and 10 µg. per ml., but no *Brucella* were cultured from the aureomycin concentration

TABLE X
The action of aureomycin on *Brucella suis*

Time interval	Control		Aureomycin (10 µg./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	AM-free subculture
30 min.	—	+++	—	+++
1 hour	—	+++	—	+++
6 hours	—	+++	—	++
24 hours	++	++++	—	+++
2 days	+++	++++	—	++++
3 days	++++	++++	—	++++
4 days	++++	++++	+	++++
6 days	++++	++++	+++	++++
10 days	++++	++++	++++	++++
14 days	++++	++++	++++	++++

effect was seen in the combination of aureomycin and normal serum having good bactericidal power (see Tables XII-XV).

The Resistance of Brucella to Aureomycin

Several unsuccessful attempts were made to isolate aureomycin-resistant variants of *Brucella*

TABLE XIII

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. II. Broth control

No. of bacteria	100	50	25	125	6.25	3.12	1.56	0.78	0.39	0	µg./ml.
150,000,000	-	-	+++	++++	++++	++++	++++	++++	++++	++++	
15,000,000	-	-	+	++	++	+++	+++	+++	+++	++++	
1,500,000	-	-	++	++	++	++	+++	+++	+++	++++	
150,000	-	-	+	+	++	++	++	++	++	+++	
15,000	-	-	-	-	+	+	+	+	+	++	
1,500	-	-	-	-	-	-	+	+	+	+	
150	-	-	-	-	-	-	-	-	-	+	
	100	50	25	125	6.25	3.12	1.56	0.78	0.39	0	µg./ml.

Aureomycin concentration

TABLE XIV

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. III. Inactivated serum

No. of bacteria	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0	µg./ml.
150,000,000	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	
15,000,000	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	
1,500,000	++	++	+++	+++	+++	+++	+++	+++	+++	+++	
150,000	++	++	++	++	++	++	+++	+++	+++	+++	
15,000	-	-	-	-	+	+	+	+	+	++	
1,500	-	-	-	-	+	+	+	+	+	+	
150	-	-	-	-	-	-	+	+	+	+	
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0	µg./ml.

Aureomycin concentration

TABLE XV

The relationship between the antibrucella activity of aureomycin and the bactericidal power of serum. IV. Convalescent brucellosis serum

No. of bacteria	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0	µg./ml.
150,000,000	++	+++	+++	+++	+++	++++	++++	++++	++++	++++	
15,000,000	+	+	++	++	++	++	+++	+++	++++	++++	
1,500,000	+	+	+	+	++	++	++	++	+++	+++	
150,000	+	+	+	+	+	+	+	+	+	++	
15,000	-	-	-	+	+	+	+	+	+	+	
1,500	-	-	-	-	-	-	-	-	-	-	
150	-	-	-	-	-	-	-	-	-	-	
	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0	µg./ml.

Aureomycin concentration

present in a large bacterial population, using the method described for the isolation of streptomycin-resistant variants. A further attempt was made to develop aureomycin-resistant strains of *Brucella* by exposing the organisms to increasing concentrations of aureomycin.

Method: A 1:100 dilution in broth of a 24-hour culture of *Br. abortus* was exposed to aureomycin in concentrations from 250 to 0.25 $\mu\text{g.}$ per ml. in the manner described for the broth dilution method of determining the sensitivity of *Brucella* to aureomycin. After incubating for 72 hours, the broth-aureomycin mixture in each tube was examined for turbidity and at the same time a loopful was streaked on tryptose agar. The turbid broth containing the highest concentration of aureomycin was diluted 1:100 and reexposed to aureomycin in the same manner. This procedure was repeated at 72-hour intervals, using as the inoculum in each test the broth from the preceding test containing the highest concentration of aureomycin permitting the multiplication of the bacteria.

Results: After 16 exposures of the strain of *Br. abortus* to aureomycin, there was no significant increase in resistance, as measured by the antibiotic necessary to inhibit growth, or by that

amount necessary to kill the bacteria (see Figure 5).

Strains of Brucella Isolated after Aureomycin Therapy

One strain of *Br. abortus* and three strains of *Br. melitensis* isolated after aureomycin therapy were tested for sensitivity to the antibiotic. There were no changes in the sensitivity to aureomycin when compared to the sensitivity of the strain isolated from the same patient before therapy.

III. CHLOROMYCETIN

The observation by Ehrlich (10) and Gottlieb (11) that chloromycetin inhibited the growth of gram-negative organisms prompted the investigation of the action of his antibiotic against *Brucella*.

In vitro Sensitivity of Brucella to Chloromycetin

Twenty-five strains of *Brucella*, including 14 strains of *Br. abortus*, 10 strains of *Br. melitensis*, and one strain of *Br. suis*, were tested for sensitivity to chloromycetin (lots No. Rx X3146 and

Repeated Exposure of *Brucella Abortus* to Decreasing Concentrations of Aureomycin

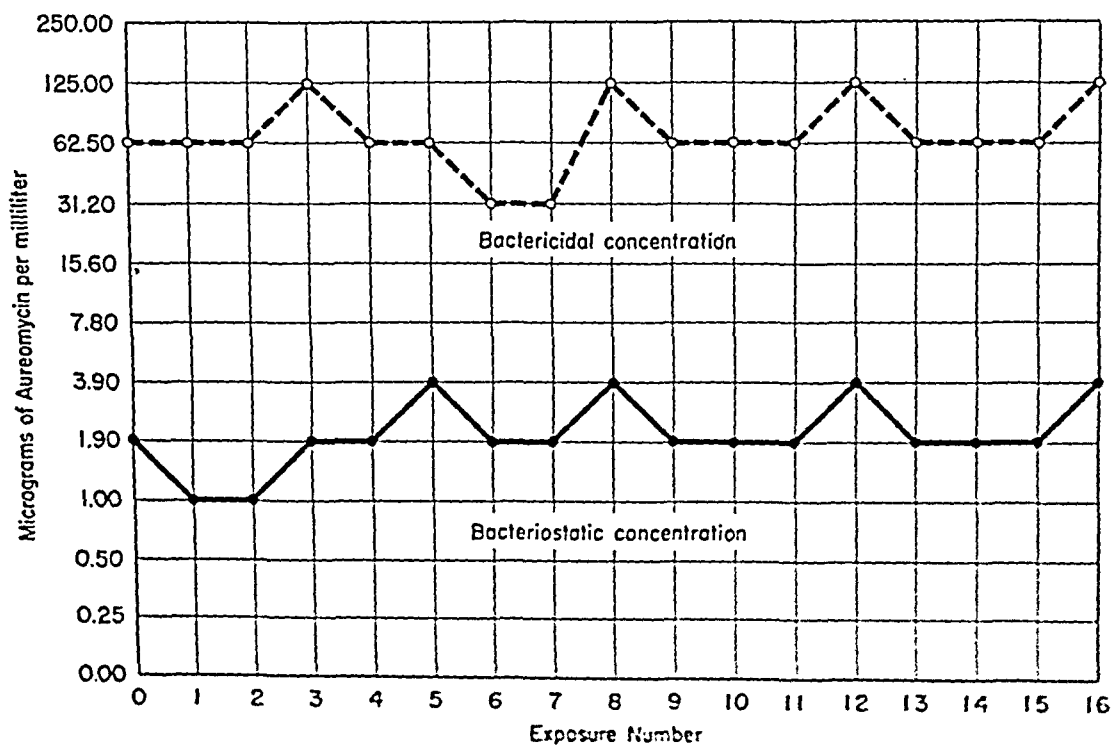


FIG. 5

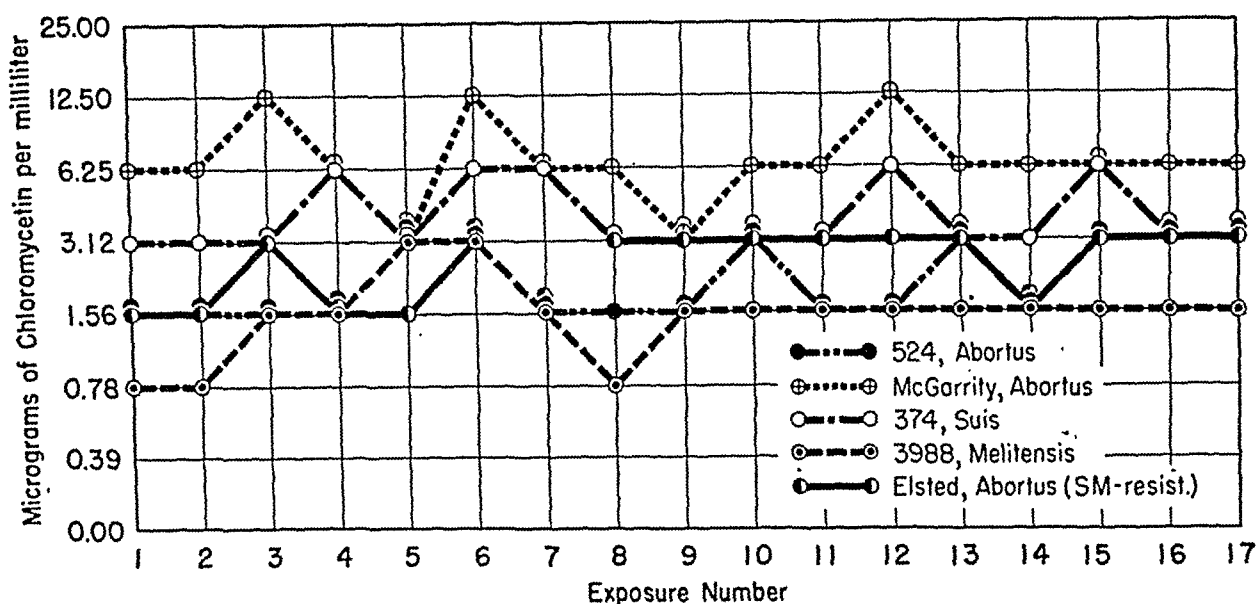
Repeated Exposure of Strains of *Brucella* to Chloromycetin

FIG. 6

120021). The method used was that described for the determination of the sensitivity of *Brucella* to aureomycin. The concentration of chloromycetin required to inhibit the multiplication of the strains of *Brucella* tested varied from 1.56 to 6.25 $\mu\text{g. per ml.}$, while the concentration required to kill the organisms was from 15.6 to 125 $\mu\text{g. per ml.}$ (see Tables XVI and XVII).

The Action of Chloromycetin on *Brucella*

The method used to study the action of chloromycetin on *Brucella* was similar to that described

for streptomycin. The concentrations of chloromycetin used were 5.0, 20, and 100 $\mu\text{g. per ml.}$ The tests revealed that there was no evidence of multiplication of *Br. abortus* when exposed to chloromycetin. Viable organisms could be recovered after 48 hours from the chloromycetin-broth mixture containing 5 $\mu\text{g. per ml.}$ for five days, from the mixture containing 20 $\mu\text{g. per ml.}$ for three days, and from the tube containing 100 $\mu\text{g. per ml.}$ for two days. The results with strains of *Br. melitensis* and *Br. suis* varied from

TABLE XVI
Sensitivity of *brucella abortus* to chloromycetin

Strain	Lowest conc. inhibiting growth $\mu\text{g./ml.}$	Highest conc. permitting growth $\mu\text{g./ml.}$	Conc. sterilizing culture at 48 hrs. $\mu\text{g./ml.}$
Lynch	1.56	0.78	15.6
Kreutz	3.12	1.56	
Archer*	3.12	1.56	
Steinbrecker	3.12	1.56	
Steinbrecker*	3.12	1.56	
Wimmer	6.25	3.12	>125
Wimmer*	6.25	3.12	
Garrity	3.12	1.56	
Garrity*	3.12	1.56	
Elsted†	3.12	1.56	
Johnson	3.12	1.56	
Nelson	3.12	1.56	
Glick	3.12	1.56	
Schneider	3.12	1.56	

* Isolated with streptomycin-sulfadiazine therapy

† Streptomycin resistant

TABLE XVII
Sensitivity of *Brucella melitensis* and *Brucella suis* to chloromycetin

Strains of <i>Brucella melitensis</i>	Minimum conc. inhibiting growth $\mu\text{g./ml.}$	Maximum conc. permitting growth $\mu\text{g./ml.}$	Conc. sterilizing culture at 48 hrs. $\mu\text{g./ml.}$
3752	3.12	1.56	125
3925	1.56	0.78	
3925*†	1.56	0.78	
2716	3.12	1.56	
3777	3.12	1.56	
3988	1.56	0.78	
3988*	1.25	0.62	
4103	1.56	0.78	
4103*	3.12	1.56	
4858	1.56	0.78	
Strain of <i>Brucella suis</i> 374	3.12	1.56	7.8

* Isolated after streptomycin-sulfadiazine therapy

† Streptomycin resistant

those obtained with strains of *Br. abortus* in that the multiplication of the former occurred in 5 μ g. of chloromycetin per ml., though the appearance of the turbidity was delayed when compared to the broth control (see Tables XVIII-XX).

The Development of Resistance of Brucella to Chloromycetin

Preliminary attempts to produce chloromycetin-resistant variants of *Brucella* have been unsuccessful.

TABLE XVIII
The action of chloromycetin on Brucella abortus

Time interval	Control		Chloromycetin (5 μ g./ml.)		Chloromycetin (20 μ g./ml.)		Chloromycetin (100 μ g./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	—	++	—	++	—	++	—	++
1 hour	—	++	—	++	—	++	—	++
3 hours	—	++	—	++	—	++	—	++
24 hours	+	++++	—	++	—	+	—	+
2 days	++	+++++	—	++	—	+	—	+
3 days	++++	+++++	—	+	—	+	—	—
4 days	+++++	+++++	—	+	—	—	—	—
5 days	+++++	+++++	—	+	—	—	—	—
6 days	+++++	+++++	—	—	—	—	—	—
7 days	+++++	+++++	—	—	—	—	—	—

TABLE XIX
The action of chloromycetin on Brucella suis

Time interval	Control		Chloromycetin (5 μ g./ml.)		Chloromycetin (20 μ g./ml.)		Chloromycetin (100 μ g./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	—	+++	—	+++	—	+++	—	+++
1 hour	—	+++	—	+++	—	+++	—	+++
3 hours	—	+++	—	+++	—	+++	—	+++
24 hours	++	+++++	—	+++	—	++	—	++
2 days	+++	+++++	—	++	—	+	—	+
3 days	+++++	+++++	—	++	—	+	—	—
4 days	+++++	+++++	+	+++	—	—	—	—
5 days	+++++	+++++	+	+++	—	—	—	—
6 days	+++++	+++++	+	+++	—	—	—	—
7 days	+++++	+++++	+	+++	—	—	—	—

TABLE XX
The action of chloromycetin on Brucella melitensis

Time interval	Control		Chloromycetin (5 μ g./ml.)		Chloromycetin (20 μ g./ml.)		Chloromycetin (100 μ g./ml.)	
	Broth turbidity	Agar subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture	Broth turbidity	Chloro-free subculture
30 min.	—	+++	—	+++	—	+++	—	+++
1 hour	—	+++	—	+++	—	+++	—	+++
3 hours	—	+++	—	+++	—	+++	—	+++
24 hours	++	+++++	+	+++++	—	+++	—	+++
2 days	+++	+++++	+	+++++	—	++	—	++
3 days	+++++	+++++	+	+++++	—	++	—	++
4 days	+++++	+++++	++	+++++	—	+	—	+
5 days	+++++	+++++	+++	+++++	—	—	—	—
6 days	+++++	+++++	+++	+++++	—	—	—	—
7 days	+++++	+++++	+++	+++++	—	—	—	—

cessful. The methods used included the exposure of a large bacterial population to concentrations of 10, 50 and 100 μg . of chloromycetin per ml., as described for streptomycin, and the repeated exposure of four strains of *Brucella* to increasing concentrations of chloromycetin (see Figure 6).

IV. OTHER ANTIBIOTICS

Eight strains of *Br. abortus* and one strain of *Br. suis* were tested for sensitivity to amorphous penicillin and to crystalline penicillin G. The broth dilution method was used. The concentration of amorphous penicillin required to prevent the multiplication of *Brucella* was found to vary from 5 to 31 units per ml., while the concentration required to sterilize the cultures was from 16 to 62 units per ml. The concentration of crystalline penicillin G inhibiting growth varied from 1.0 to 62 units per ml., whereas the sterilizing concentration varied from 12.5 to 250 units per ml.

The sensitivity of six strains of *Br. melitensis* to bacitracin was determined. Concentrations greater than 10 units per ml. were required to prevent multiplication of the bacteria. Two strains of *Br. abortus*, one of *Br. melitensis*, and one of *Br. suis* were tested for sensitivity to the antibiotic Q-19 (Research No. 8836-1). The concentration which inhibited growth of *Br. abortus* was 62.3 units per ml., though concentrations greater than 125 units per ml. were required to prevent the growth of the strains of *Br. melitensis* and *Br. suis*.

DISCUSSION

No naturally occurring strains of *Brucella* resistant to streptomycin, dihydrostreptomycin, aureomycin, or chloromycetin were found. On a weight basis, aureomycin was the most active agent against *Brucella*, streptomycin and dihydrostreptomycin were slightly less active, and chloromycetin was the least active antibrucella agent. There was very little strain or species variation in the sensitivity of *Brucella* to streptomycin, dihydrostreptomycin, or aureomycin, but there was considerable variation from strain to strain in chloromycetin sensitivity.

Streptomycin and dihydrostreptomycin were rapidly bactericidal in their action against *Brucella*. Aureomycin and chloromycetin, on the

other hand, acted primarily by preventing the multiplication of the bacteria, and concentrations much higher than have been reported in the blood were necessary to kill *Brucella*.

Strains of *Brucella* resistant to streptomycin and dihydrostreptomycin could be readily produced *in vitro* by culturing a large bacterial population in the presence of the antibiotics, suppressing the sensitive organisms in the population and allowing the normally present resistant variants to multiply. Similar attempts to produce strains resistant to aureomycin and chloromycetin were unsuccessful. Attempts to adapt strains of *Brucella* to growth in the presence of high concentrations of aureomycin and chloromycetin by repeated exposure of the organisms to the antibiotics were also largely unsuccessful. One would be tempted to predict from these results that the development of resistance of *Brucella* in human infections to streptomycin and dihydrostreptomycin might occur rapidly and frequently, but resistance to aureomycin and chloromycetin would occur infrequently and progress very slowly.

The problem of the development of resistance of *Brucella* as a cause of failure in the therapy of human infections was investigated by determining the sensitivity of strains of *Brucella* recovered from patients after therapy. Of approximately 26 patients with bacteriologic proved brucellosis due to *Br. abortus* treated at the University of Minnesota Hospitals, there have been six relapses following combined sulfadiazine and streptomycin therapy, while approximately 30 of 65 patients treated in Mexico with infections due to *Br. melitensis* subsequently had proven bacteremia. There were two relapses out of 12 patients with infections due to *Br. abortus* following aureomycin therapy, while *Brucella* were recovered after treatment from five out of 26 patients with infections due to *Br. melitensis*. The clinical use of chloromycetin in brucellosis is now being studied.

Of the 13 cultures of *Brucella* isolated after combined streptomycin and sulfadiazine therapy, one strain of *Br. melitensis* showed increased resistance to streptomycin. Another strain of *Br. abortus*, recovered after therapy with streptomycin alone, had marked resistance to the drug. There was no evidence of an increase in the normally present streptomycin-resistant variants in the strains which failed to respond to therapy.

Three strains of *Br. melitensis* and one strain of *Br. abortus*, cultured from human subjects, after aureomycin therapy, showed no changes in sensitivity to aureomycin.

One may conclude from these studies that the relapse of brucellosis following combined streptomycin and sulfadiazine or aureomycin therapy is rarely due to a change in the sensitivity of the bacteria to streptomycin or aureomycin. The evidence suggests that the strains cultured following therapy, with the exception of the two streptomycin-resistant strains, are identical to the strains isolated from the same patients before therapy. The most likely explanation for these therapeutic failures is that the antibiotics do not actually come into direct contact with all the *Brucella* harbored by the host within tissue cells, within granulomata present in the reticuloendothelial system, or within other walled-off foci (12). These and other possibilities are now being investigated.

SUMMARY

1. Strains of *Br. abortus*, *Br. suis*, and *Br. melitensis*, isolated from patients before antibiotic therapy, were sensitive *in vitro* to concentrations of streptomycin and dihydrostreptomycin of 1.0 to 2.5 $\mu\text{g. per ml.}$, to aureomycin in concentrations of 0.6 to 1.5 $\mu\text{g. per ml.}$, and to concentrations of chloromycetin of 1.56 to 6.25 $\mu\text{g. per ml.}$

2. The action of streptomycin against *Brucella* was bactericidal, while aureomycin and chloromycetin were bacteriostatic in their action.

3. Resistance of *Brucella* to streptomycin and dihydrostreptomycin could be developed readily *in vitro*, but increased resistance to aureomycin and chloromycetin was not demonstrated.

4. The study of 13 strains of *Brucella* isolated after combined streptomycin and sulfadiazine therapy revealed no change in their sensitivity to streptomycin with the exception of one strain of *Br. melitensis*, which increased in resistance from 2.5

to 50,000 $\mu\text{g. of streptomycin per ml.}$ One strain of *Br. abortus* isolated after a course of streptomycin alone increased in resistance from 1.0 to 10,000 $\mu\text{g. of streptomycin per ml.}$ Three strains of *Br. melitensis* and one strain of *Br. abortus* recovered from patients after aureomycin therapy showed no change in sensitivity to aureomycin.

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ANTIGENIC AND ENZYME SYSTEM CHANGES IN BETA HEMOLYTIC STREPTOCOCCI RESISTANT TO PENICILLIN, STREPTOMYCIN, BACITRACIN AND AUREOMYCIN^{1, 2, 3, 4}

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Previous reports from this laboratory (1, 2) have presented preliminary data suggesting that there are antigenic and enzyme system changes in beta hemolytic streptococci resistant to penicillin and streptomycin. These data were all qualitative and were unconfirmed by quantitative methods.

The present study is a continuation of this work. The same strains of group A and C streptococci were made resistant separately to bacitracin and aureomycin. Representative members of each group, resistant to all four antibiotics, were studied for alterations in: 1) streptolysin S; 2) streptokinase; 3) proteinase; 4) ribonuclease activity.

That such variations might be expected was first suggested by the observation that reversible changes in the hemolytic behavior of streptococci from beta to alpha or gamma type of hemolysis occurred when the organisms were growing on maximal concentrations of either penicillin or streptomycin.

MATERIALS AND METHODS

Strains of Streptococci

Seven group A strains and three group C strains isolated in 1946-1947 from throats of patients with acute upper respiratory infections were employed throughout the study. The strain designations for the group A or-

ganisms were: S₁, S₂, S₃, S₄, S₅, S₆, and U₃; and, for the group C strains, U₁, U₂, and U₃.

Development of Resistance

The method employed for inducing penicillin and streptomycin resistance has been described (1, 2). A similar technique was used for inducing bacitracin and aureomycin resistance. Briefly, this consisted of daily transfer of organisms from a blood agar plate containing the maximal quantity of antibiotic that would permit growth to a new series of plates containing graded amounts of antibiotic. For the streptomycin, bacitracin, and aureomycin series a total of 40 such transfers were made, and for the penicillin series 60 transfers.

Streptolysin S Production

The method described by Todd (3) was employed for all tests of streptolysin S production. Thirty ml Todd-Hewitt medium containing 20% normal horse serum inactivated at 56° C. for 30 minutes were inoculated with 1 ml of an 18 hour streptococcal broth culture in Todd-Hewitt medium prepared with 2% Pfanstiehl peptone. After incubation at 37° C. for 12 hours, the cultures were thoroughly chilled in an ice water bath, centrifuged at 3,000 rpm for 20 minutes and the supernate filtered through Sela candles (No. 02 porosity with a maximum pore size of 0.85 micron). Only cultures of resistant strains with densities that matched that of the control strain, i.e., the parent organism transferred 40 times on plain blood agar medium, were employed. The densities were determined in a Coleman Junior Spectrophotometer. Filtrate dilutions ranging from 1:4 to 1:200 were prepared in cold physiological saline so that, after the addition of 0.5 ml of a 5% suspension of fresh triple-washed rabbit RBC's, the final volume was 2.0 ml. The hemolytic end point was considered to be the dilution which showed approximately 50% hemolysis after incubation for 30 minutes in a 37° C. water bath. The lysin then was titrated against anti-streptolysin S rabbit serum (4).

Streptokinase Activity

The method employed for determining the streptokinase activity of the supernate of the broth culture was essentially that described by Kaplan (5). The fibrinogen⁵

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³ Aureomycin was supplied by the Lederle Laboratories Division, American Cyanamid Co., through the Antibiotics Study Section of the National Institutes of Health.

⁴ Bacitracin was supplied by the Commercial Solvents Corporation, through the Antibiotics Study Section of the National Institutes of Health, and by Dr. Frank L. Melroy of Columbia University.

⁵ The fibrinogen employed was Fraction I of the plasma proteins prepared by the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., from blood collected by the American Red Cross, and generously supplied by Dr. Edwin J. Cohn and Dr. John T. Edsall.

was used in a concentration of 600 mg dissolved in 100 ml phosphate-buffered physiological saline at pH 7.4. Purified human thrombin was prepared so that each ml of buffered saline contained approximately 3 units. The fibrinolytic titer was expressed as the reciprocal of the dilution in which there was complete dissolution of the clot after incubation in a water bath for one hour at 37° C.

Proteinase Activity

The streptococcal proteinase activity in broth cultures was measured by the method described by Elliott and Dole (6). Briefly, this consisted of observing the rapidity and degree of coagulation of a skimmed milk-thioglycollate substrate by sterile broth culture filtrates. Twenty-four hour cultures of streptococci in Todd-Hewitt broth containing 2% Pfanstiehl peptone were filtered through Selas candles (No. 02 porosity). Maintaining sterility, two-fold dilutions of filtrate were made in phosphate buffer pH 7.0. The milk-thioglycollate substrate was added with an automatic pipette, and the mixture was incubated at 37° in a water bath. The test was read at one, three, five, and nine hours and the activity expressed as the reciprocal of the dilution showing coagulation at nine hours.

Ribonuclease Activity

The ribonuclease activity of culture supernates was determined by the turbidimetric method described by McCarty (7). The substrate, yeast ribonucleic acid, was purified by the method of Kunitz (8). The optical densities were determined on a Coleman Junior Spectrophotometer at a wave length of 425 mμ, at five minute intervals over a 30 minute period.

RESULTS

The group A strains, in general, developed little resistance to penicillin and aureomycin, and

TABLE I
Antibiotic resistance acquired by beta hemolytic streptococci

Group	Strain	Fold change after 40 transfers on			
		Penicillin medium*	Streptomycin medium	Bacitracin medium	Aureomycin medium
A	S ₁	4	140	100	2
A	S ₂	2	70	20	6
A	S ₃	17	140	40	6
A	S ₄	0	140	100	6
A	S ₅	4.5	240	40	5
A	S ₆	4	180	13	6
A	U ₅	6	70	8	8
C	U ₁	16	3,000	20	10
C	U ₂	14	600	300	60
C	U ₃	4	200	300	20

* After 60 transfers

TABLE II

Streptolysin-S production of groups A and C beta hemolytic streptococci

Group	Strain	Hemolytic units per ml.				
		Control*	Pen.† res.	Strep.‡ res.	Bac.‡ res.	Aureo.‡ res.
A	S ₁	75	35	—	20	75
A	S ₂	35	10	20	35	35
A	S ₃	75	10	13	75	50
A	S ₄	75	—	35	75	100
A	S ₅	20	4	—	10	—
A	U ₅	120	30	—	—	120
C	U ₁	75	13	75	20	35
C	U ₂	120	13	—	45	120
C	U ₃	75	20	75	50	75

* Control organism is the parent strain after 40 transfers on plain blood agar.

† Penicillin-resistant organism is one that has been transferred 60 times on medium containing increasing concentrations of penicillin.

‡ Streptomycin-, bacitracin-, and aureomycin-resistant organisms are those that have been transferred 40 times on media containing increasing concentrations of each antibiotic.

considerable resistance to streptomycin and bacitracin. The group C strains showed similar group behavior to each antibiotic. The results are given in Table I.

The rate at which a given strain of beta hemolytic streptococcus acquired resistance to an antibiotic was not uniform in the case of penicillin, streptomycin, bacitracin, and aureomycin. For example, strain S₄ after 60 serial transfers on penicillin medium showed no change in sensitivity, after 40 serial transfers on aureomycin medium only slight resistance, but considerable resistance to streptomycin and bacitracin after 40 subcultivations on media containing these antibiotics.

Streptolysin S production was reduced for each group A organism after acquiring either penicillin or streptomycin resistance. The same strains after acquiring bacitracin or aureomycin resistance maintained the same level of hemolysin production as the control strain. The group C organisms behaved somewhat differently in their production of streptolysin S. The three penicillin-resistant and three bacitracin-resistant organisms all produced less hemolysis than the control strains. The streptomycin-resistant and aureomycin-resistant strains showed little if any change. The results are given in Table II.

derstanding of the mechanism of action of acquired antibiotic resistance. Further, it is not known why penicillin and streptomycin resistance appears to alter the behavior of streptococci to a much greater extent than does either bacitracin or aureomycin resistance. The degree of resistance for streptomycin and bacitracin and that for penicillin and aureomycin are comparable.

The variables of medium, time, temperature and amount of growth so far have been eliminated as the probable explanation for the reduced enzymatic production by the resistant variants. Each series of organisms was grown for the same period of time, at the same temperature, in a single lot of medium and on the same day. In every instance, the density of the resistant variants was matched turbidimetrically with that of the parent and control organisms.

The possibility exists, of course, that during the numerous serial transfers another strain of streptococcus was introduced through air-borne contamination or through a change in serial order of several strains on a single plate. This is partially answered by the fact that all of the resistant variants for all four antibiotics were of the same group as the parent organism. Strains that were ungroupable after acquired penicillin resistance have not been included in this study. Final proof will come from typing the parent and resistant strains. This is now in progress.

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PATTERNS OF BACTERIAL RESISTANCE TO PENICILLIN, AUREOMYCIN, AND STREPTOMYCIN¹

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During the last few years genetic research with bacteria has made significant progress, and ample evidence has accumulated to show that the hereditary mechanism operating in bacteria may be similar to that in higher organisms. It has been demonstrated that hereditary changes, comparable to mutations, occur in bacteria, and that these cover a range of types similar to those found in fungi, where genetic analysis is possible (1). Furthermore, an association resembling linkage in higher organisms has been observed in bacteria (2).

Experimental evidence indicates that changes in bacteria from sensitivity to resistance to penicillin and streptomycin originate as mutations, and that these antibiotics act only as selective agents which eliminate the sensitive bacteria and thus allow the resistant mutants, which are always present in any large population, to multiply (3). Experiments have revealed that the development of high resistance follows a definite pattern, which appears to be characteristic for each antibiotic and which is not determined by the bacteria involved. Detailed analyses of the pattern of streptomycin resistance have been made by me with *Escherichia coli* and *Staphylococcus aureus*, and by Dr. V. Bryson (unpublished) with *Mycobacterium ranae*. This work has revealed a remarkable similarity of behavior in these species of bacteria. Quantitative studies of penicillin resistance have been made only with *Staphylococcus aureus* (4, 5), but the results of other investigators, who have studied various phases of the action of penicillin with a wide variety of species, do not suggest that the pattern of resistance to penicillin is different in other bacteria from that analyzed with *Staphylococcus*.

At present two patterns of resistance have been established, the penicillin pattern and the streptomycin pattern. A "stepwise" development of re-

sistance is common to both, but they differ significantly in the manner in which high-level resistance is arrived at. In the case of penicillin, the first-step resistant mutants are very uniform in their degree of resistance, which is only slightly higher than that of the original strain. Additional mutations occurring in such first-step mutants result in bacteria possessing a higher (second-step) degree of resistance, and in a similar manner third-step and still higher resistance develops. Always the variation in degree of resistance among mutants of the same step is slight. This uniformity constitutes the most striking feature of the penicillin pattern; and it explains the stepwise increase in resistance. Because a significant degree of resistance can be attained only by additional mutation in an already mutant bacterium, at least two mutations in the same individual are required to bring it about. Since the mutation rate is low, however—about 1×10^{-8} per bacterium per generation—the chances for simultaneous occurrence of such double mutations are extremely low (1×10^{-16} , or practically nil), so that a large number of first-step resistant bacteria must first be present in order that a second-step mutant may occur. In other words, higher resistance may be attained only in successive steps, and no step may be skipped in this process.

The streptomycin pattern of resistance differs from the penicillin pattern in that the variation between first-step mutants is very great. They range from individuals only slightly more resistant than the original bacteria to those having complete resistance to streptomycin. Thus, although higher resistance may be attained in successive steps, as with penicillin, it may also arise in a first-step mutant.

I have attempted to analyze the resistance pattern for aureomycin, but this work has been complicated by the mode of action of that antibiotic on *Escherichia coli*, the organism used for the experiments. It was found that aureomycin, in addition to killing some of the bacteria, suppresses

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TABLE I

Per cent of bacteria appearing as colonies after certain incubation periods when plated in broth-agar containing various concentrations of aureomycin

Concentration $\mu\text{g/ml}$	Hours incubation				
	24	72	96	120	144
	<i>Per cent colonies</i>	<i>Per cent colonies</i>	<i>Per cent colonies</i>	<i>Per cent colonies</i>	<i>Per cent colonies</i>
Control	100				
1	0	75			
5	0	0.12	70		
10	0	0	0.15	29	
15	0	0	0	0	4.1
20	0	0	0	0	3.5
30	0	0	0	0	0.7

division in the survivors. Since the aureomycin loses some of its potency during an experiment, these suppressed bacteria begin to divide again whenever the concentration of antibiotic on the plate is lowered to a certain point (Table I). If a large number of bacteria is plated, some will begin to grow earlier than others. In the anticipation that these might be more resistant to aureomycin, they were used to establish strains, which were tested for resistance; and from these a further selection of early growers was made. In this way strains that appeared to be more resistant to aureomycin were obtained. One such strain, obtained through five selections, when plated in broth-agar containing 10 μg of aureomycin per ml, gave 50 per cent survival, with colonies appearing after 48 hours of incubation. Under similar conditions, the original strain gave 29 per cent survival, the colonies appearing only after 120 hours of incubation.

These results show definitely that the pattern of aureomycin resistance is different from that of streptomycin resistance, in that highly resistant mutants do not appear in one step. It may be similar to the penicillin resistance pattern, but analysis to determine this has not been carried through because of complications introduced into the experiments by the loss of potency of aureomycin.

The genetic mechanism responsible for both the penicillin and the streptomycin resistance patterns may be explained by the assumption that several genes govern the reactions that determine sensitivity or resistance, and that the set of genes affecting penicillin resistance is different from the set of

genes affecting streptomycin resistance. If any one of these genes should mutate, the bacterium in which such a mutation occurs and the strain developed from that bacterium will be more resistant to the respective antibiotic than was the original parent strain. Such a strain is what we have called a "first-step resistant strain."

The fact that first-step penicillin-resistant strains are fairly uniform in degree of resistance is consistent with the assumption that all genes affecting resistance to penicillin have a similar potency, so that the effect of mutation is the same regardless of which of the genes happens to mutate. According to this hypothesis, there is still present in a first-step resistant strain a number of unmutated genes that affect resistance. Mutation of any of these produces a second-step resistant strain, which possesses a higher degree of resistance than the first-step strain. Similarly, by mutation of another gene in a second-step resistant strain, a still higher degree of resistance is attained, characteristic of the third-step resistant strain; and by further repetition of the process a very high degree of resistance may be reached.

The observed behavior of resistance to streptomycin also can be explained by assuming the existence of several genes determining such resistance. Unlike the genes for penicillin resistance, however, these differ greatly from one another in potency. If a gene of low potency mutates, the first-step resistant strain will have a low degree of resistance, but if mutation occurs in a highly potent gene, the first-step resistant strain will be highly resistant. Consequently, considerable variation in degree of resistance is to be expected between first-step strains; and for the same reason a highly resistant strain may be obtained either in one step, by selection of a highly resistant first-step mutant, or in several steps, by selection of mutants of low resistance values.

The assumption that several genes are responsible for resistance is not unusual; a similar assumption, involving the presence of about 20 genes, has already been made to explain the complex situation observed in a study of resistance of *E. coli* to several bacteriophages (6). Also, experiments now under way in my laboratory indicate that several genes are involved in changes of *E. coli* from streptomycin sensitivity to strepto-

mycin resistance and dependence, as well as in re-versions from dependence to sensitivity.

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THE PARTIAL PURIFICATION AND PROPERTIES OF ANTIBIOTIC SUBSTANCES FROM THE SWEET POTATO PLANT (*IPOMOEA BATATAS*)^{1, 2}

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The dramatic chemical and therapeutic successes of certain antibiotics, notably the penicillins and streptomycin from *Penicillium* and *Actinomyces* molds, have spurred the search among other microorganisms and, more recently, among angiosperms for additional substances possessing antibacterial and antifungal properties.

Within the past few years it has been repeatedly shown by many investigators that some plant juices and extracts of dried whole plants and plant parts contain various kinds and intensities of antibacterial and antifungal activity (1-8).

In connection with investigations in this laboratory it has been previously reported (7) that the autoclaved expressed juice of the sweet potato plant (*Ipomoea batatas*) inhibited cultures of several plant-wilt producing *Fusaria*. The present investigations have confirmed and elaborated upon this early observation.

It is the purpose of this paper to present a procedure for obtaining crude and partially purified preparations of antibiotic substances from the sweet potato plant, to discuss some of the chemical, physical, bacteriological, and mycological properties of the various active fractions, and to present some of the speculative implications of this work in connection with the problems associated with some human, and possibly animal, diseases.

To date, no pure antibacterial or antifungal compound has been isolated. Since the crude extracts and some of the partially purified fractions were considered relatively impure and were found to contain both inhibitory and some stimulatory material, it was not deemed advisable, as yet, to

set up an arbitrary unit of activity. It will thus be appreciated that the results reported here have only qualitative significance and that the interpretations of the results must be regarded as tentative.

EXPERIMENTAL

Two varieties³ of sweet potato plants were used throughout this work. One variety was *Fusarium*-wilt-resistant (designated *Res.*), whereas the other variety was *Fusarium*-wilt-susceptible (designated *Susc.*).

Using the familiar disc and cup-plate assay method with slight modifications to be described, the test microorganisms listed in Table I were employed in this work.

Method of assay

A. Into individual, sterile, 90-mm. Petri dishes were poured base layers of 20 ml. of agar as follows:

1. Nutrient agar—for all bacteria except *Mycobacterium phlei*
2. Glycerol agar—for *M. phlei*
3. Czapek-dextrose agar (3) for the *Fol.* and *Fob.*

B. The inocula were prepared and introduced onto the plates as follows:

1. For all bacteria except *M. phlei*:

Two-tenths of a milliliter of a 24-hr. nutrient broth culture was added to 5 ml. of warm (42° C) nutrient agar. This seed layer was then evenly poured over the agar surface of a prepared Petri dish. The agar was allowed to harden, and the covered plate was then incubated for one-half hour at 37° C before "spotting" or the addition of test solution.

2. For *M. phlei*:

Five milliliters of an homogenized and filtered four-day-old culture were added to 50 ml. of warm (42° C) glycerol agar. Four milliliters of this suspension was evenly flooded over the agar surface of a Petri dish. The covered dish was then incubated for one hour at 37° C before "spotting."

¹Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

²Report of a study made under the Research and Marketing Act of 1946.

³*Ipomoea batatas*. No. 153655, *Fusarium*-wilt-resistant variety, and Puerto Rico Unit 1, *Fusarium*-wilt-susceptible variety. Both varieties were grown at Beltsville, Maryland, and obtained from the Bureau of Plant Industry, Soils, and Agricultural Engineering.

TABLE I

Microorganisms used in assays of sweet potato extracts

Microorganism and source*	Gram-stain
<i>Fusarium oxysporum</i> f. <i>batatas</i> Wr. (Snyder and Hansen) (D F7357e) (designated <i>Fob.</i>)	—
<i>F. oxysporum</i> f. <i>lycopersici</i> (Snyder and Hansen) (W No. R-5-6) (designated <i>Fol.</i>)	—
<i>Escherichia coli</i> (Migula) Castellani and Chambers (NRRL No. B210)	+
<i>Staphylococcus aureus</i> Rosenbach (NRRL No. B313)	—
<i>Serratia marcescens</i> Bizio (ATCC no. 60)	—
<i>Xanthomonas translucens</i> f. sp. <i>Hordei-avenae</i> Hagborg (ATCC No. 9000)	+
<i>Mycobacterium phlei</i> Lehmann and Neumann (ATCC No. 355)	(Acid-fast)
<i>Sarcina lutea</i> Schroeter (ATCC No. 272)	+
<i>Bacillus cereus</i> Fr. and Fr. (C No. 401)	+
<i>Rhodococcus roseus</i> Winslow and Rogers (ATCC No. 177)	g. var.
<i>Bacillus subtilis</i> Cohn [†] (NRRL No. 558)	+

* ATCC, American Type Culture Collection; NRRL, Northern Regional Research Laboratory; C, H. R. Curran, Bureau of Dairy Industry, Washington, D. C.; D, S. P. Doolittle, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland; W, F. L. Wellman, formerly Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.

3. For *Fol.* and *Fob.*:

Six milliliters of sterile distilled water were used to wash down a four-day-old potato-dextrose slant of the organism. This aqueous suspension of spores was filtered through a thin, sterile, cotton plug to remove bits of the mycelium which would tend to give rise to irregular growth, if plated. One and three-tenths ml. of this filtrate were added to 100 ml. of liquid Czapek-agar (42° C), and 4 ml. of the resulting suspension were used to flood the prepared plate evenly. After hardening of the agar, the covered plate was incubated for 24 hours at 28° C before the addition of the test solutions.

The technique of "spotting" consisted of saturating, by imbibition, a standard filter-paper assay disc (S & S 740-E) with the test solution and immediately placing the disc on the inoculated surface of the prepared Petri dish. If the antibiotic was dissolved solely in an organic solvent, the filter-paper assay disc was saturated and placed in a vacuum desiccator until the solvent had evaporated. The dried disc was then saturated with water and immediately placed on the inoculated surface. The covered dish was then inverted and incubated under the same optimum conditions for the growth of the organism. Biological activity was determined by the presence or absence, and the relative sizes, of zones of inhibition or stimulation.

Preliminary survey of sweet potato plants

Direct hot and cold aqueous extractions of the dried and ground whole Res. sweet potato plant resulted in

solutions which generally stimulated the growth of the characteristic test microorganisms, *Fol.*, *Fob.*, *Escherichia coli*, and, to a slighter degree, *Staphylococcus aureus*. No effect was noticed with *M. phlei*. Similar results were noted for aqueous extracts of Res. tubers, leaves, stems, and Susc. tubers and stems. The water extracts of the Susc. leaves and of the roots, as well as the water-insoluble extraction residues of all plant parts except Susc. tubers, very slightly inhibited *Fob.* Direct aqueous extractions were abandoned as being unsatisfactory.

When portions of dried and ground Res. and Susc. sweet potato plant parts were individually extracted with methanol, the extract reduced to dryness by removal of the methanol, and the resulting residue taken up in water, the aqueous solutions thus produced, in marked contrast with the direct aqueous extracts, inhibited cultures of microorganisms previously unaffected or largely stimulated. Only the extracts of Susc. stems inhibited *M. phlei*. In almost all cases stimulation of some microorganisms was apparent, concurrently with concentric zones of inhibition.

Of the various plant parts extracted and assayed, the leaves and stems of both the plant-wilt-resistant and the susceptible varieties seemed to contain at least as much, if not more, inhibitory material than did the roots and tubers. Extracts of Susc. stems appeared to be the most active of all.

Procedure for obtaining crude active extracts

Weighed samples of dried and ground Susc. sweet potato stems were extracted with methanol for eight hours in a Soxhlet extraction apparatus. The dark green methanolic extracts were then concentrated to dryness *in vacuo*, and the residues were thoroughly extracted, portionwise, with sufficient warm distilled water to make the final volumes of clear, reddish-brown aqueous extract

TABLE II

Assay of crude active extracts* obtained from Susc. sweet potato stems

Test organism	Aq.-Conc.	MeOH.-post-Aq.-Conc.†
<i>Fol.</i>	+++	+++
<i>Fob.</i>	+++	++
<i>Escherichia coli</i>	+	0
<i>Staphylococcus aureus</i>	+++=	-+
<i>Serratia marcescens</i>	+++=	0
<i>Mycobacterium phlei</i>	+++	0
<i>Bacillus subtilis</i>	+++=	0
<i>Xanthomonas translucens</i>	+++=	0
<i>Sarcina lutea</i>	+++=	()
<i>Bacillus cereus</i>	+++=	()
<i>Rhodococcus roseus</i>	+++=	()

* +++, complete inhibition; ++, partial inhibition; +, slight indication of inhibition; =, complete stimulation; ++, partial stimulation; -, slight indication of stimulation; 0, no effect; (), no assay.

† All the organisms listed were not used against all 13 preparations of each concentrate.

‡ Dilutions were made such that no more than three drops of organic solvent were added to each 10 ml. of agar on any assay plate.

represent 1 gram of original stem material per ml. The aqueous solutions thus prepared were considered "concentrates" and designated "Aq.-Conc." They were found to contain most of the antibacterial activity. The non-water-soluble residues were taken up in minimal amounts of methanol and designated "MeOH-post-Aq.Conc." The latter methanolic solutions, so prepared, contained most of the antifungal activity.

The approximate average assay results of 13 separate preparations of Aq.-Conc. and MeOH-post-Aq.Conc. are summarized in Table II.

Partial purification of active concentrates

Measured weights and volumes of the clear, reddish-brown aqueous concentrate, Aq.-Conc., were stirred batchwise for 15 min. at 25° C with from 2.0% to 2.5% (by weight) of activated carbon (Darco G-60) * while the pH of the solution was controlled in the range of 4.0 to 4.5 by the dropwise addition of 1 *N* hydrochloric acid solution. The carbon was filtered and washed with several small portions of distilled water, and the washings were added to the deep orange-brown filtrate (effluent). The carbon cake was then transferred to a beaker and to it was added a volume of distilled water—equal to that of the original sample of Aq.-Conc. used. The mixture was again stirred for 15 min. while the pH of the solution was adjusted and controlled at from 7.0 to 7.5 by the dropwise addition of 1 *N* ammonium hydroxide solution.

The carbon was then filtered from the solution, washed with several small portions of distilled water, and discarded. The washings were added to the sparkling, clear, pale-yellow eluate.

RESULTS

The average assay results obtained from three separate batchwise adsorption-elution runs are summarized in Table III.

These results seem to indicate that the water-soluble material that stimulates fungi may be separated from the materials that inhibit bacteria. The material inhibitory toward Gram-positive bacteria appeared to be concentrated in the neutral to slightly basic eluate.

Columnar adsorption using an intimate mixture of equal parts of activated carbon (Darco G-60) and Kieselguhr (Hyflo Super-Cel) resulted in irreversible adsorption of most of the active material in the original concentrates. No significant quantity of active material could be eluted, at reasonable pH values, with water, methanol, or various water-methanol mixtures. Percolation of

* The mention of this and other commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

TABLE III

*Assay of partially purified aqueous concentrates obtained from Susc. sweet potato stems**

Organism	Aq.-Conc.	Effluent†	Eluate‡
<i>Fol.</i>	=	=	0
<i>Fob.</i>	≡	≡	0
<i>Escherichia coli</i>	+ -	+ -	0
<i>Staphylococcus aureus</i>	+++ -	+++ -	+++
<i>Xanthomonas translucens</i>	+++ -	+++ =	+++ -
<i>Sarcina lutea</i>	+++	+	+++
<i>Mycobacterium phlei</i>	+++	0	+++
<i>Bacillus cereus</i>	+++	0	+++

* The symbols are the same as those used in Table II.

† Average pH value adjusted to 6.8.

‡ Average pH value adjusted to 6.6.

active concentrates through Kieselguhr (Hyflo Super-Cel) filter beds containing no carbon, likewise resulted in irreversible adsorption and consequent loss of activity.

The use of chromatographic column containing activated carbon (Darco G-60) with no siliceous filler produced indeterminate results not inconsistent with those in Table III. However, these attempts presented such mechanical difficulties that the method was abandoned. A search is now being conducted for a physically and chemically suitable and non-adsorbent filler for columnar use with finely divided carbon.

Preliminary work with synthetic ion-exchange resins, such as the "Amberlites," has been started. As yet, insufficient data have been accumulated to warrant drawing any general conclusions other than that they offer some promise for purification and concentration of the active materials.

Some properties of active preparations

The control of pH to within rather narrow limits appeared to be a critical factor for adsorption and elution of antibacterial substances. Except at the optimum pH values, an overall distribution of non-selective inhibitory and stimulatory material occurred. This was accompanied by some loss of total activity. All the active material was found to be soluble in polar, and insoluble in non-polar, solvents. The antifungal material appeared to be very soluble in methanol but not appreciably water-soluble, whereas all the antibacterial and all the stimulatory material appeared to be very soluble in water.

Samples of active preparations seemed to retain their activity after being heated for as long

as eight hours at 100° C. Samples refrigerated for one week at 4° C showed no perceptible diminution of activity. The results of several investigations of serial dilutions on agar slants, made with different preparations of Aq.-Conc. against *M. phlei*, demonstrated that dilutions as low as 0.05 ml. Aq.-Conc. per ml. of total solution completely inhibited the organism. This inhibition was entirely retained after 10 days. The inhibitory material was considered bactericidal to *M. phlei*, in the aforementioned concentration, since it was found impossible to initiate growth of the organism upon transfer of the attenuated culture to a fresh slant.

DISCUSSION AND CONCLUSIONS

Crude extracts of sweet potato plant parts contained a conglomerate mixture of non-selective inhibitory and stimulatory activity. Since it has been found possible to separate, from crude extracts, individual aqueous and methanolic fractions showing, respectively, the predominate bacterial activity and the major portion of antifungal activity (Table II), and since it has been further found possible to obtain fractions of the aqueous concentrate, Aq.-Conc., which exhibit selective inhibition to Gram-positive microorganisms (Table III), it has been postulated that at least two discrete inhibitory materials and one stimulatory material may be present in the total crude concentrates.

Although no sweeping conclusions can be drawn at this time, the method successfully employed for the partial purification of the aqueous concentrates indicated that the antibiotic factor or factors responsible for the activity against Gram-positive microorganisms may be polar in nature. If the inhibitory effect against Gram-positive organisms resulted from the action of an individual substance, then, as was indicated by the results of serial dilution investigations with *M. phlei*, this substance may be a bactericide in relatively low concentrations.

It is fully appreciated by the authors that the present investigations were largely exploratory in nature and that the results reported represent work done on crude extracts and partially purified fractions. However, the partial isolation of active material selectively inhibitory, *in vitro*, to Gram-

positive microorganisms, particularly *M. phlei*, encourages speculation concerning the possible therapeutic applicability of this material in the treatment of some human, and possibly animal, mycobacterial infections. It is further realized that before any thought of possible therapeutic use of this active material can be seriously entertained, investigations in addition to those now in progress must demonstrate satisfactory and desirable kinds and degrees of activity and potency *in vivo*, stability under various conditions, and, among other properties, either no toxicity or sufficiently low toxicity to render feasible any administration.

SUMMARY

1. Aqueous and methanolic extracts of dried whole sweet potato plants and dried and ground plant parts were found, by the familiar disc and cup plate assay method, to contain both antibiotic and stimulatory activity when tested against certain Gram-negative and Gram-positive bacteria (including one acid-fast form) and two forms of plant-wilt fungi.

2. Of the plant parts from wilt-resistant and wilt-susceptible varieties that were extracted and assayed, the stems of the wilt-susceptible variety appeared to be the most desirable source of antibiotic substances.

3. By use of batchwise and columnar adsorption and elution techniques, it was possible to separate partially the antifungal from the antibacterial activity and to prepare partially purified concentrates exhibiting selective inhibitory, and possibly bactericidal, activity against Gram-positive microorganisms.

4. Preliminary evidence is cited for postulating the existence of at last three discrete biologically active materials in the sweet potato plant.

5. Some of the chemical, physical, bacteriological, and mycological properties of the active solutions are discussed.

6. Preliminary antibacterial and antifungal spectra of the various active extracts and fractions are presented.

ACKNOWLEDGMENT

The assistance of Mrs. Barbara Black in conducting antibiotic assays, and of Mr. Francis Guay for the prep-

aration of some sweet potato extracts, is greatly appreciated.

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THE PARTIAL PURIFICATION AND PROPERTIES OF ANTIBIOTIC SUBSTANCES FROM THE BANANA (*MUSA SAPIENTUM*)^{1,2}

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Recent investigations have shown that extracts of higher plants contain substances which inhibit the growth of bacteria and fungi (1). In a preliminary survey conducted by this laboratory, approximately 40% of the extracts from the 80 plants tested exhibited either antibacterial or antifungal activity or both. Among the plants tested was the banana. Bananas have been used clinically for dietary purposes and for some intestinal disorders (2), but their beneficial effect has never been attributed to the presence of antibiotic substances. The banana skin has been referred to as nature's bacteria-proof wrapper and investigations have shown that when bananas were immersed in fluids containing cultures of known organisms there was no evidence of a penetration into the interior (3).

The purpose of this paper is to present the partial antibiotic spectrum of extracts obtained from the pulp and skins of green, naturally ripened, and ethylene-ripened bananas, and from banana leaves and petioles, and to describe the partial purification of these antibiotic substances.

EXPERIMENTAL

Preparation of extracts

Fresh undried plant material was extracted with water. Plant material previously dried at 85° C in a forced-draft oven and ground to pass a medium Wiley mill screen was extracted with methanol or petroleum ether (b.p. 35-60° C). Aqueous, methanol, and petroleum-ether extracts were concentrated until each milliliter of solution was equivalent to 1 gm. of the plant material. In some experiments the organic-solvent extracts were evaporated to dryness and sufficient water to make each milliliter of solution equivalent to 1 gm. of original plant material was then added to the residues. Frequently, not all of the substance dissolved by organic solvent was

soluble in water. In these cases the water-soluble portion was removed, and the water-insoluble portion was tested separately. The pH of all aqueous solutions was determined and then adjusted to 6-7 for assay, although no appreciable difference in the size of inhibition zones was noted when the aqueous solutions were not adjusted from their normal pH of 4.0 to 5.5.

Method of assay

Sterile, 90 mm. petri dishes, containing 20 ml. of solidified agar media, were warmed to 45° C and flooded with 4 ml. of agar, seeded with bacterial cells or fungus spores. In the cylinder cup-plate method the plant extracts were pipetted into the cups which had been placed on the inoculated agar, and in the paper disc method the discs were immersed in the plant extract long enough to become saturated with the solution and then dried in a vacuum desiccator. Before they were placed on the inoculated agar the discs were moistened with distilled water. The agar plate was incubated for a fixed period of time at a temperature that was optimum for the growth of the organism. The zones of inhibition around the cups or discs were measured to obtain a relative indication of the amount of antibiotic agent in the extract. See (1) for source of organisms.

Antibiotic activity of crude extracts

Aqueous extracts of banana leaves and petioles showed no detectable antibiotic activity. The water-soluble portion of the substance extracted by methanol exhibited no appreciable antifungal activity, whereas the water-insoluble portion inhibited the growth of the fungus *Fusarium oxysporum* f. *lycopersici*.

Some antibacterial activity was detected in the methanol extracts of both plant parts. The water-soluble portion of the substance extracted from leaves by methanol inhibited *Xanthomonas translucens* f. sp. *hordei-avenae* and *Rhodococcus roseus* and partially inhibited *Sarcina lutea* and *Serratia marcescens*, whereas the same portion from the methanol extract of petioles inhibited *Staphylococcus aureus*, *S. lutea*, *Mycobacterium phlei*, and *R. roseus*.

A comparison of the antifungal and antibacterial activities of the water-soluble substance in methanol extracts of the pulp and skins of green, naturally ripened, and ethylene ripened bananas² is shown by Table I.

² The green, naturally ripened and ethylene ripened bananas were obtained from the Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Maryland.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² Report of a study made under the Research and Marketing Act of 1946.

TABLE I

Comparison of the activities* of extracts obtained from the pulp and skins of green, naturally ripened, and ethylene-ripened bananas

Organism†	Skins			Pulp		
	Green	Naturally ripened	Ethylene-ripened	Green	Naturally ripened	Ethylene-ripened
<i>Fusarium oxysporum</i> f. <i>lycopersici</i>	29+	25+	29+	17-, 25+	28+	32.5+
<i>Escherichia coli</i>	27(-)	25+, 28-	22+, 28-	23(-)	25+, 33-	28+, 34-
<i>Staphylococcus aureus</i>	18(+), 23(-)	25+, 28(-)	19+, 25(+), 30(-)	20(+)	27+, 32-	27+, 35-
<i>Serratia marcescens</i>	26(+), 33(-)	27+, 34-	32+, 37-	24(+), 33(-)	28+, 35-	30+, 37-
<i>Mycobacterium phlei</i>	18(-)	+	19+	0	+	18+
<i>Bacillus subtilis</i>			19+, -, 33(+), (-)			17+, -, 39(+), 42(-)
<i>Sarcina lutea</i>			29+			25+
<i>Rhodococcus roseus</i>			28+, (-)			23+
<i>Xanthomonas translucens</i> f. sp. <i>hordei-avenae</i>			30+			24+

* The numbers refer to the diameter of the zone in millimeters; for instance, 25+, 28- means an inhibition zone of 25 mm. and a stimulation zone of 3 mm. beyond the 25 mm. zone. +, inhibition; (+), partial inhibition; -, stimulation; (-), partial stimulation; 0 = no effect.

† See previous paper (1) for source of organisms.

RESULTS

Antifungal activity was exhibited by all six extracts. There was very little, if any, measurable antibacterial activity in either the pulp or skins of green bananas, but there was appreciable antibacterial activity in the pulp and skins of ripe bananas. In some instances the inhibition zones were surrounded by zones of stimulation. This may be due either to the carbohydrates which are present, mainly as sugars, in ripe bananas (4) or to a subminimal amount of antibiotic at the outer edge of the zone of inhibition or to a combination of both factors. The partial inhibition zones resulting from extracts of the pulp and skins of green bananas may be explained by the fact that some of the bananas were partly ripe. It appears that the antibacterial activity of bananas is due to the formation of inhibitory compounds during ripening. Petroleum ether did not extract any of the active substance from either the pulp or the skins.

Agar slants of the water-soluble portion of a crude methanol extract of the skins indicate that the substance at a concentration of 0.25 gm. of

dried plant material per ml. of medium is fungistatic to *F. oxysporum* f. *lycopersici*, *Trichophyton mentagrophytes*, *Monosporium apiospermum* and *Blastomyces dermatitidis*.

Fractionation and partial purification

Dried skins (1,325 gm.) from ethylene-ripened and naturally ripened bananas were combined and extracted, batchwise, with 10 successive 1,500-ml. portions of hot methanol, and the combined extracts were subsequently concentrated to approximately 2 liters. When this concentrate was cooled to -20° C, some resinous material was precipitated. The supernatant methanolic solution was filtered and concentrated under reduced pressure to approximately 1,300 ml. so that each ml. of solution represented about 1.0 gm. of dried skins. This solution showed a notable increase in antifungal activity, producing an inhibition zone of 62 mm. against *F. oxysporum* f. *lycopersici*, which is more than twice the diameter of the zone produced by solutions from which the resinous material had not been separated, but it did not show any appreciable change in antibacterial activity. As-

says of ethanol solutions of the resinous material showed slight inhibitory activity toward bacteria and some stimulatory activity toward *F. oxysporum* f. *lycopersici*.

Thus far, chromatographic adsorption of the antibiotic substances in methanol extracts, and in the petroleum-ether-soluble portion of methanol extracts, by activated alumina has not proved very satisfactory. Activated carbon (Darco G-60)⁴ did not adsorb the antibiotic substances from an aqueous extract (pH 5.0) of the methanol concentrate.

Low pressure distillation of a methanol concentrate of ripe banana skins produced a small amount of liquid from which a few crystals have been obtained. During the distillation, at 2-mm. pressure, the tendency to foam was controlled by cautious heating. A few drops of distillate were collected at a pot temperature of 180–190° C, and a few additional drops as the pot temperature rose to 250° C. Both distillates were readily soluble in ethanol, but upon the addition of a few drops of water the solutions became turbid. Crystals formed in each solution when cooled in the refrigerator for a few days. At the time of writing, the crystals had not been tested for antibiotic activity, but the original solution from which the crystals separated had been found to exhibit both antifungal and antibacterial activity (Table II). The physical and chemical properties of the crystalline substance have not yet been determined.

TABLE II

Activity of the distillate obtained from ripe banana skins

Organism*	Inhibition zones mm.
<i>Fusarium oxysporum</i> f. <i>lycopersici</i>	29
<i>Escherichia coli</i>	35
<i>Staphylococcus aureus</i>	30
<i>Sarcina lutea</i>	42
<i>Xanthomonas translucens</i> f. sp. <i>hordei-avenae</i>	41
<i>Mycobacterium phlei</i>	35
<i>Bacillus cereus</i>	33

* See previous paper (1) for source of organisms.

DISCUSSION

The results show that all four parts of the banana plant tested contain some extractable antibiotic

agent(s). Since the pulp and skins of both green and ripe bananas showed antifungal activity, and the pulp and skins of only ripe bananas showed antibacterial activity, it seems likely that there are at least two separate antibiotic agents in ripe bananas. Further, the antibacterial substance appears to be a product formed during the ripening process. It is interesting to note that a vacuum-distillable substance was obtained from ripe banana skins which had both antifungal and antibacterial activity.

Adsorption on alumina and on activated charcoal produced no positive results, but these procedures have not yet been fully investigated.

The *in vitro* antifungal and antibacterial activity of the various parts of the banana plant warrants further investigation to isolate, identify and characterize the active substance or substances. Because of the *in vitro* effectiveness of these principles, toxicological investigations are now in progress.

SUMMARY

1. Extracts of banana leaves were found to be only slightly antibacterial, whereas extracts of stems inhibited the growth of two Gram-positive, one acid-fast Gram-positive, and one Gram-variable bacterium.

2. Extracts of the pulp and skins of green bananas inhibited only the fungus *F. oxysporum* f. *lycopersici*.

3. Extracts of the pulp and skins of ripe bananas effectively inhibited *F. oxysporum* f. *lycopersici*, *Escherichia coli*, *Staph. aureus*, *S. marcescens*, *M. phlei*, *Bacillus subtilis*, *S. lutea*, *R. roseus*, and *X. translucens* f. sp. *hordei-avenae*.

4. A substance distillable at low pressure effectively inhibits *F. oxysporum* f. *lycopersici*, *E. coli*, *Staph. aureus*, *S. lutea*, *M. phlei*, *Bacillus cereus*, and *X. translucens* f. sp. *hordei-avenae*.

5. The results indicate that the banana plant contains one or more substances with therapeutic possibilities provided their toxicity and *in vivo* activity are favorable.

ACKNOWLEDGMENT

The assistance of Mrs. Barbara Black in conducting antibiotic assays, and of Mr. Francis Gossy for the preparation of some banana extracts, is greatly appreciated.

⁴ The mention of this and other commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

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ANTIBIOTIC STUDIES ON AN EXTRACT FROM *LEPTOTAENIA MULTIFEDA*^{1, 2, 3}

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Numerous surveys (1-8) have demonstrated the wide occurrence of antibiotically active materials in higher plants. In a recent investigation Matson and Flowers (9) examined 440 plants found in Utah and neighboring states. Certain of these plants have long been used for medicinal purposes by both American Indians and white men. Among the families which appeared to contain a higher percentage of species and genera containing antibiotic substances was the *Umbelliferae* family. Matson and Flowers found in preliminary experiments that an aqueous extract of the root and leaves of *Leptotaenia multifida*, commonly known as "Indian carrot," inhibited growth of *Micrococcus aureus* and *Escherichia coli*. The root of *L. multifida* has been used by the Gosiute Indians in the form of a paste for application upon wounds, cuts or bruises where the skin was broken (10). This dressing was particularly applied in case of infection.

Some of the preliminary work of the present paper was reported by Ravve (11). At about the same time a publication by Carlson and Douglas (12) described the antibiotic activity of oil fractions obtained from the root of a related plant, *Leptotaenia dissecta*. Further work on the extraction and purification of an oil from the root of *L. multifida* and a study of its antibiotic properties is herein described.

METHODS

Extraction and purification. *L. multifida* was collected on the foothills of the Wasatch Range northeast of Salt Lake City, Utah. The plants were stored in a refrigerator

until used. Among the several methods studied for the extraction of oil from the root, the following represents a preferred procedure. The water-washed and peeled root was macerated in a meat grinder, placed in a flask and covered with ethyl acetate. After 24 to 48 hours at room temperature the mixture was filtered, and the solvent was removed under reduced pressure from the yellow filtrate. The recovered ethyl acetate was suitable for reextraction purposes. The extraction was conducted at room temperature in order to minimize any tendency toward loss of activity through polymerization. The extraction of a given batch of root was repeated three times. This procedure yielded approximately 9% of crude extract by weight of the cleaned, peeled, wet root of *L. multifida*.

The crude extract was washed 10 or more times with water. This treatment gave material which will be designated as extract purified by water washing.

A more complete purification procedure involved treatment with sodium bisulfite. The crude oil was first washed thoroughly with a dilute, aqueous solution of sodium bicarbonate and then thoroughly washed with water. To 150 ml. of a saturated solution of sodium bisulfite in 95% ethanol was added a solution of 25 ml. of oil in 150 ml. of absolute alcohol. The resulting precipitate was filtered and washed with several portions of ethanol. There was no visible evidence of unreacted oil. The bisulfite addition complex was added to a two phase mixture of 30% aqueous acetic acid and petroleum ether (b.p. 30-60° C.). The petroleum ether, containing the liberated oil, was washed 10 or more times with water and dried over anhydrous sodium sulfate. Solvent removal under reduced pressure left the regenerated oil in 50 to 60% yield based on the crude oil. The resulting product was dissolved in alcohol-free benzene and was passed through a column packed with Magnesol 4-Celite⁴ (five to one, by weight) to remove polymerized material. Solvent removal left a light yellow oil in approximately 50% yield based on the bisulfite purified extract. Elution of the column with 200 to 1 benzene-ethanol (by volume) followed by solvent removal gave a brown viscous oil.

The use of ethylene glycol as a carrier in distillations under reduced pressures appeared to be useful if small quantities only of the oil were desired. At 60° C. and several microns pressure ethylene glycol and the oil, which were immiscible, distilled in the volume ratio of about five to one, respectively.

⁴ A product of Westvaco Chlorine Products Co., South Charleston, West Virginia.

⁵ No. 535. A product of Johns-Manville Co., New York, New York.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

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Agar plate assay. To 250 ml. of cooled melted agar medium was added 5 ml. of an 18 to 24 hour broth culture of the test organism. An amount of 20 ml. of the seeded agar was poured into each petri plate and allowed to harden at room temperature. A sterile filter paper disc 10 mm. in diameter was immersed in the material to be assayed. Excess liquid was allowed to drain, and the disc was placed in the center of the poured plate. Plates were observed after 18 to 24 hours incubation at 37° C. The diameter of the zone of inhibition was measured to the nearest millimeter and noted as complete or partial. The figures given are averages of three or more plates. Dilutions of the materials tested were made in sterile mineral oil, Saybolt viscosity 335-350 at 38° C. Mineral oil controls were made with each organism. Penicillin G sodium at a concentration of 10^{-2} Gram per ml. of aqueous solution was used for comparative purposes. In addition Duracillin diluted with peanut oil to contain the same number of penicillin units per ml. as the aqueous solution was employed.

Broth tube assay. To 4.5 ml. of serial dilutions of the oil in sterile mineral oil was added 0.05 ml. of a one to 100 dilution of an 18- to 24-hour broth culture of the test organism. The tubes were shaken to distribute the organisms thoroughly through the mineral oil. After one-fourth, one-half, one, eight, and 24 hours, loopfuls (diameter of loop 4 mm.) of the suspensions were withdrawn from each tube and added to sterile broth. All tubes were observed for growth 48 hours after inoculation.

Toxicity assay. The animals used were 20-Gram, male, Swiss, white mice eight to ten weeks old. All mice received one subcutaneous injection. In certain instances the oils were diluted with sterile peanut oil to facilitate injection. The crude extract was free of ethyl acetate. Sterile peanut oil was used as the control.

RESULTS

Ethyl acetate extraction of the washed and peeled macerated root of *L. multifida* provided a rapid and convenient means of obtaining a yellow, water insoluble oil in high yield.

The crude extract was tested *in vitro* for antibacterial properties against 14 representative Gram-positive and Gram-negative organisms (Table I). Complete or partial inhibition of growth was exhibited with ten organisms including all of those belonging to the Gram-positive group. In most cases in which the oil was active, it was effective at a concentration of 10^{-3} in mineral oil. At comparable concentrations (10^{-2}) penicillin and the crude oil were roughly equally effective against the organisms susceptible to both agents.

The extract purified by water washing was also evaluated in agar plate tests (Table II). On the whole and particularly at the lower concentrations, thorough washing with water appeared to have resulted in greater activity. The aqueous extracts from the washing treatments did not show any appreciable activity against Gram-positive organisms.

The effectiveness of the water-washed extract against three organisms in nutrient broth tests was also determined (Table III). These results were comparable with those obtained in agar

TABLE I
Activity of a crude extract

Organism	Diameter of zone of inhibition in mm.								
	Straight	Conc. in mineral oil						Penicillin G, 10^{-2} , in	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Peanut oil	Water
<i>Micrococcus aureus</i>	80	55	47	25	14	12	n	20	24
<i>Corynebacterium diphtheria</i>	42	34	28	18	14	n	n	40	43
<i>Diplococcus pneumoniae III</i>	37	40	28	22	n	n	n	17	22
<i>Streptococcus pyogenes</i>	33	44	30	25	15	n	n	27	32
<i>Bacillus subtilis</i>	24	32	28	20	n	n	n	27	33
<i>Vibrio cernae</i>	45	33	35	26	n	n	n	41	50
<i>Neisseria catarrhalis</i>	37	39	29	22	12	n	n	26	31
<i>Escherichia coli</i>	40p	37p	15p	n	n	n	n	19	25
<i>Pseudomonas aeruginosa</i>	33p	18p	n	n	n	n	n	n	n
<i>Proteus vulgaris</i>	17p	13p	n	n	n	n	n	33p	37p
<i>Salmonella schottmuelleri</i>	n	n	n	n	n	n	n	27	33
<i>Aerobacter aerogenes</i>	n	n	n	n	n	n	n	13	18
<i>Klebsiella pneumoniae</i>	n	n	n	n	n	n	n	14	17
<i>Serratia marcescens</i>	n	n	n	n	n	n	n	n	n

n=no inhibition
p=partial inhibition

TABLE II
Activity of extract purified by water washing

Organism	Diameter of zone of inhibition in mm.								
	Straight	Conc. in mineral oil						Penicillin G, 10 ⁻³ , in	
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	Peanut oil	Water
<i>Micrococcus aureus</i>	78	58	29	24	14	11	n	20	24
<i>Corynebacterium diphtheria</i>	56p	48p	39p	30p	18p	n	n	40	43
<i>Diplococcus pneumoniae III</i>	51	44	36	28	18	14	n	17	22
<i>Streptococcus pyogenes</i>	39	24	29	27	21	11	n	27	32
<i>Bacillus subtilis</i>	51	39	34	22	13	n	n	27	33
<i>Vibrio comma</i>	61	43	32	31	14	n	n	41	50
<i>Neisseria catarrhalis</i>	74	67	39	35	15	13	n	26	31
<i>Escherichia coli</i>	45p	17p	14p	11p	n	n	n	19	25
<i>Pseudomonas aeruginosa</i>	24p	14p	12p	n	n	n	n	n	n
<i>Proteus vulgaris</i>	15	14	13	n	n	n	n	33p	37p
<i>Klebsiella pneumoniae</i>	n	n	n	n	n	n	n	14	17
<i>Serratia marcescens</i>	12	15	13	n	n	n	n	n	n

n—no inhibition

p—partial inhibition

TABLE III
Activity of extract purified by water washing as determined in nutrient broth

Time in hours	Conc. in mineral oil																	
	<i>Micrococcus aureus</i>						<i>Escherichia coli</i>						<i>Streptococcus pyogenes</i>					
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
½	—	—	—	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+
1	—	—	—	—	+	+	+	+	+	+	+	+	—	—	—	+	+	+
8	—	—	—	—	+	+	+	+	+	+	+	+	—	—	—	+	+	+
24	—	—	—	—	+	+	—	+	+	+	+	+	—	—	—	—	+	+

— indicates no growth

+ indicates growth

TABLE IV
Activity of bisulfite treated extract after and prior to passage through Magnesol-Celite

Organism	Diameter of zone of inhibition in mm.					
	Straight	Conc. in mineral oil				
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
<i>Micrococcus aureus</i>	—	78(45)	61(20)	32(15)	17(14)	11(11)
<i>Streptococcus pyogenes</i>	90(37p)	85(25p)	30(27p)	30(19p)	20(15p)	6p(11p)
<i>Bacillus subtilis</i>	—	82(28)	68(21)	38(18)	17(12)	13(n)
<i>Vibrio comma</i>	85(85p)	83(77p)	58(42p)	30(26p)	19(16p)	6p(14p)
<i>Escherichia coli</i>	34p(n)	16(n)	14(n)	13p(n)	n(n)	—
<i>Serratia marcescens</i>	23p(n)	23p(n)	15p(n)	14p(n)	n(n)	n(n)

Figures in parentheses are activities prior to passage through Magnesol-Celite.

— not done

n—no inhibition

p—partial inhibition

TABLE V
Effect of the oil on acid-fast organisms

Organism	Diameter, zone of inhibition in mm.				
	Crude extract	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
<i>Mycobacterium lacticola</i>	55	49	41	22	12
<i>M. phlei</i>	57	42	36	17	12
<i>M. tuberculosis</i> , var. <i>hominis</i>	40	40	22	15	11

plate tests in that the oil was much more active against the Gram-positive organisms.

Regeneration of the oil from the crystalline sodium bisulfite addition complex was accomplished by treatment with aqueous acetic acid. Although the reaction mixture was cooled, some polymerization appeared to have occurred as evidenced by the increased viscosity of the resulting oil. When this material in benzene solution was passed through a column packed with Magnesol-Celite (five to one, by weight), an oil with greater fluidity was obtained upon removal of solvent. The activity of this material was determined (Table IV) and found to be significantly greater than either the crude or the water-washed extract. The dark viscous oil adsorbed on the column exhibited but little activity against *M. aureus*.

The crude oil was found to be effective against both pathogenic and non-pathogenic acid-fast organisms (Table V). In all cases zones of complete inhibition were obtained at a concentration as low as 10⁻⁴ in mineral oil.

TABLE VI
Toxicity of extract toward mice

	Dose in ml.	No. of mice	Fatalities
Crude	0.025*	3	0
	0.05†	3	3
Water washed	0.30	2	0
	0.50	2	0
Bisulfite purified	0.05†	3	0
	0.18‡	3	0
Passed through column	0.18	2	0
Adsorbed on column	0.15	2	0
Peanut oil control	0.50	3	0

* Diluted to 0.05 ml. with peanut oil

† Diluted to 0.10 ml. with peanut oil

‡ Diluted to 0.27 ml. with peanut oil

Crude, water-washed, and bisulfite-purified specimens of the oil were tested for toxicity toward mice. Bisulfite-purified extract passed through Magnesol-Celite and material adsorbed on the column were also evaluated. The low toxicity of the water-washed and bisulfite-purified oils was indicated by the fact that subcutaneous injections of from 0.9 to 2.5 % of body weight of the mice did not noticeably affect their behavior (Table VI). Tolerance of the crude extract before washing with water was considerably lower.

The heat sensitivity of the crude oil was indicated by the reduction of activity against *M. aureus* and also by the increase in viscosity when the oil was maintained at 100° C. or higher for one hour (Table VII). Oil obtained by steam distillation of the crude extract at atmospheric pressure (640 mm.) likewise showed reduced activity against

TABLE VII
Effect of heat on a crude extract

Conc. in mineral oil	Activity against <i>Micrococcus aureus</i> Diameter of zone of inhibition in mm.			
	Control unheated	1 Hour at °C.		
		100	150	200
10 ⁻¹	47	40	15	27
10 ⁻²	36	19	18	11
10 ⁻³	25	15	11	n
10 ⁻⁴	21	11	n	n
10 ⁻⁵	14	n	n	n

this organism. No appreciable loss of activity against *M. aureus*, however, was noted when the crude oil was heated in ethyl acetate at 69° C. for two hours.

DISCUSSION

The fact that an antibiotically active oil can be extracted from *L. multifida* in high yield has greatly facilitated the investigation of this material since ample quantities for the desired laboratory studies were readily obtained.

While the oil was quite effective in agar plate tests against certain Gram-negative organisms such as *Vibrio comma* and *Neisseria catarrhalis*, it appeared to be much more generally active against members of the Gram-positive group. This was indicated by the complete inhibition obtained even at concentrations of 10⁻³ with all Gram-positive organisms used. These included

M. aureus, *Corynebacterium diphtheria*, *Diplococcus pneumoniae* III, *Streptococcus pyogenes*, and *Bacillus subtilis*. These tests suggest that the oil diffused through an essentially aqueous medium. A water-insoluble antibiotic of this nature might afford effective action for a comparatively long period of time *in vivo*. The oil was also effective *in vitro* against *Mycobacterium tuberculosis*, var. *hominis* and the two other acid-fast organisms tested. The results indicate the desirability of *in vivo* tests, which are in progress.

Washing the crude extract with water provided an effective means of removing material which was somewhat toxic to mice. The fact that subcutaneous injections of water-washed oil equivalent to 2.5% of the weight of the mice did not produce any deaths, or even any noticeable difference in behavior, suggested that the active material was only slightly if at all toxic to mice when administered in this way.

Heat stability tests showed that the oil underwent polymerization readily at 100 to 200° C. with a gradual loss in activity against *M. aureus*. This tendency toward polymerization upon heating complicated the purification of the oil since conventional distillation procedures even at low pressures and in the presence of antioxidants were not feasible. Distillation in the presence of immiscible liquids such as water or ethylene glycol offered a means of obtaining small quantities of distilled oil. A purification procedure involving regeneration of the oil from a sodium bisulfite addition complex, however, was found to be a convenient means of obtaining an oil of greatly enhanced antibacterial activity.

The oil, obtained by ethyl acetate extraction of the root of *L. multifeda*, appeared to be quite different in certain respects from the oil fractions Carlson and Douglas (12) obtained by the steam distillation of the root of *L. dissecta*. Both of these plants belong to the *Umbelliferae* family. The yellow oil obtained from *L. dissecta* was reported to decolorize upon standing in air and to be bactericidal against *E. coli* in a short time in broth tests. This behavior was clearly in contrast with that shown by the oil from *L. multifeda*, which showed no tendency toward decolorization even after several weeks in air, and which was only slightly effective against *E. coli* in broth tests and then only after long contact. Further, the active

oil obtained by Carlson and Douglas was described as being heat stable while the extract of *L. multifeda* undergoes polymerization at temperatures of 100° C. or higher with a concomitant reduction in activity.

Major emphasis thus far has been placed on the extraction and purification of the oil with a view to devising a satisfactory procedure for obtaining the active material in reasonably pure form. In preliminary chemical studies the oil was found to decolorize readily aqueous potassium permanganate and bromine in carbon tetrachloride. This behavior along with other results suggests that the antibiotic may be an unsaturated carbonyl compound containing only carbon, hydrogen, and oxygen. Further chemical studies are in progress.

SUMMARY

1. A light yellow, water-insoluble oil possessing antibiotic properties has been obtained in high yield by the ethyl acetate extraction of the macerated root of *L. multifeda*.

2. The oil showed considerable activity *in vitro* against the five Gram-positive organisms and also against certain of the nine Gram-negative bacteria tested.

3. Penicillin G and the crude or the water washed extract at concentrations of 10^{-2} were roughly comparable in agar plate tests in antibacterial activity against the Gram-positive and Gram-negative organisms sensitive to both.

4. Complete inhibition of acid-fast organisms, including *M. tuberculosis*, var. *hominis*, by the extract was observed at a concentration as low as 10^{-4} in mineral oil.

5. A procedure involving regeneration of the sodium bisulfite addition complex of the oil resulted in a purified material having substantially enhanced bactericidal activity.

6. Water washed or bisulfite purified extract showed no visibly noticeable toxic effect in mice when relatively large doses were given subcutaneously.

7. Preliminary chemical studies suggest that the oil may be an unsaturated carbonyl compound containing only carbon, hydrogen and oxygen.

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FACTORS INFLUENCING THE ANTIBIOTIC ACTIVITY OF LUPULON^{1, 2, 3}

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Lupulon is one of the two lipid-soluble antibiotics prepared from hops (*Humulus lupulus* L.) (1, 2). Michener *et al.* reported antifungal activity for both lupulon and humulon. Salle *et al.* (3) confirmed Shimwell (4), that Gram-positive organisms are more susceptible to lupulon than are Gram-negative bacteria. Chin *et al.* (5) reported its antituberculous activity both *in vitro* and *in vivo*. These findings and its relatively low toxicity (6) indicated that lupulon may be an effective chemotherapeutic agent. It becomes, therefore, desirable to investigate some important factors which may influence its activity either *in vitro* or *in vivo*.

MATERIALS AND METHODS

Crystalline lupulon used throughout this experiment was prepared at the Western Regional Research Laboratory, Albany, California. One % solution can be made in alkaline water at pH around 11.5 or by dissolving the crystals first as 10% solution in ethyl alcohol and then diluting up to volume with propylene glycol. The antibiotic activity has been found to be the same whether the solution was first made in alkaline water or in propylene glycol. The latter solution has a pH at 4.8 and has been used, freshly prepared with aseptic precautions, as the stock for further dilutions in alkaline water, serum, or culture media.

Staphylococcus aureus (FDA No. 209), *Mycobacterium phlei*, and *Mycobacterium tuberculosis* H37Rv were used in the experiments. Unless otherwise mentioned, veal-glucose broth (7) was used for growth of and tests on *Staph. aureus*, and Dubos and Davis fluid medium (8) for the mycobacteria. The Coleman junior spectrophotometer was used for turbidimetric determinations of the growth of organisms.

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RESULTS

Effect of size of inoculum. The effect of the size of inoculum of *Staph. aureus* on the antibiotic activity of lupulon was studied with the serial dilution method. Two-fold dilutions of the antibiotic were made in 0.5 ml. of the medium in nine 10-cm. test tubes, starting from a concentration of 200 µg./ml. A tenth tube containing the same amount of the medium served as the control in a series. Each tube was then inoculated with 1.5 ml. of a diluted 24-hour culture. Each of four different dilutions, namely, 1:100, 1:1000, 1:10,000, and 1:1,000,000, was inoculated to one series of tubes. The tubes were incubated at 37° C. and observations were made four, 24, and 48 hours later. The results are shown in Table I where even a faint growth was recorded positive.

Apparently the end point of no growth varied with the concentrations of the inoculum and also the time when the observation was made. Repeated experiments showed that observations at four hours after inoculation gave consistent end point at 1:640,000 or 1.56 µg./ml. in the series where the concentration of the inoculum was 1:1,000 of a 24-hour culture. This size of inoculum and time of incubation have been adopted in later experiments where serial dilution method was used.

Effect of constitution of medium. The serial dilution method was used to determine the bacteriostatic level of lupulon against *Staph. aureus* in three different media. These media,⁵ namely,

⁵ The compositions of these media are given below:

Modified Dubos and Davis medium:

Casein hydrolysate	1.0 gm.
Na ₂ HPO ₄ ·12H ₂ O	6.3 gm.
KH ₂ PO ₄	1.0 gm.
Na ₂ citrate·2H ₂ O	1.5 gm.
MgSO ₄ ·7H ₂ O	0.6 gm.
Distilled water to	1000.0 ml.
Beef heart infusion broth:	
Beef heart	5.0 gm.
Difco nutrient	1.0 gm.

TABLE I

The effect of size of inoculum of *Staph. aureus* on its growth in various concentrations of lupulon at 37° C.

Concentration of the inoculum	Hours of incubation	Concentration of lupulon, $\mu\text{g./ml.}$									
		50.0	25.0	12.5	6.25	3.12	1.56	0.78	0.39	0.20	0
1:1,000,000	4	—	—	—	—	—	—	—	—	—	—
	24	—	—	—	—	—	+	++	+++	+++	+++
	48	—	—	—	—	+	++	+++	+++	+++	+++
1:10,000	4	—	—	—	—	—	—	+	+	+	+
	24	—	—	—	—	—	++	+++	+++	+++	+++
	48	—	—	—	—	+	+++	+++	+++	+++	+++
1:1,000	4	—	—	—	—	—	—	+	+	+	+
	24	—	—	—	—	—	+	++	+++	+++	+++
	48	—	—	—	—	++	+++	+++	+++	+++	+++
1:100	4	—	—	—	—	+	+	+	+	+	+
	24	—	—	—	—	++	+++	+++	+++	+++	+++
	48	—	—	—	—	++	+++	+++	+++	+++	+++

modified Dubos and Davis medium, a beef heart infusion, and veal-glucose broth, had been shown by turbidimetry to support the growth of *Staph. aureus* to different extents. It was found, however, that the growth was completely inhibited by lupulon at the same concentration, 1.56 $\mu\text{g./ml.}$, no matter in which medium this antibiotic was incorporated.

Effect of pH

Studied with Staph. aureus. Veal-glucose medium was titrated to pH's 7.1, 7.6 and 8.1. For each pH duplicate series of six tubes were used. Into each of the six tubes in a series, 3 ml. of the medium was dispensed, containing an appropriate amount of lupulon so that after the inoculum was added to it the final concentrations of lupulon were 0, 0.2, 0.4, 0.6, 0.8, and 1.0 $\mu\text{g./ml.}$ An inoculum consisted of 3 ml. of 1:50 dilution of a 24-hour culture so that the final concentration of the bacteria in each tube was 1:100 of the culture. The growth in each tube was measured turbidimetrically after four hours incubation at 37° C. The results, as shown in Figure 1, indicated greater inhibitory power of lupulon at lower pH.

Plate-well assay was performed by following Pratt and Dufrenoy's method (9), with some

modifications. The inoculum of *Staph. aureus* of the top agar was 2%. The inhibition zones were measured after overnight incubation at 37° C., and the staining processes with 1% potassium ferricyanide and 1% ferric sulfate were applied only when necessary. Dilutions of lupulon were made in alkaline water adjusted to pH's 7, 8, 10, and 11.5. The inhibition zones produced by 50, 20, 10, and 5 $\mu\text{g./ml.}$ were fairly consistently 28, 24, 21, and 18 mm. for respective concentrations at all pH's.

Studied with M. phlei. Dubos medium used in this experiment was titrated to pH's 5, 6, 7, and 8. The concentrations of lupulon in duplicate series of tubes were 0, 30, 35, 40, 45, 50, 55, 60, 70, and 80 $\mu\text{g./ml.}$ The inoculum was made so that the final concentration of bacteria in a tube was 1:100 of a 48-hour culture. The end points, taken as the concentration in which no growth occurred after 24 hours incubation at 37° C., were 50 $\mu\text{g./ml.}$ for pH 7 and 8, and 40 $\mu\text{g./ml.}$ for pH 5 and 6.

Studied with M. tuberculosis (H37Rv). The experiment was performed in a similar manner to that with *M. phlei*. The concentrations of lupulon in a series of tubes were 0, 5, 10, 15, 20, 25, 30, and 40 $\mu\text{g./ml.}$, and the inoculum was 0.05 ml. of 14-day culture to a volume of 5 ml. of the medium. End points were taken at the end of 72 hours: At pH 7 and 8 it was 25 $\mu\text{g./ml.}$; at pH 6, 15 $\mu\text{g./ml.}$ The organisms did not grow well at pH 5.

Effect of NaCl

Studied with Staph. aureus. The veal-glucose medium contains 0.5% NaCl. The salt concen-

NaCl	5 gm.
Distilled water	1000.0 ml.
Veal-glucose broth:	
Veal infusion	500.0 gm.
Bacteriological peptone	10.0 gm.
NaCl	5.0 gm.
Dextrose	1.0 gm.
Distilled water	1000.0 ml.

tration was also increased to 1 and 2%. Since NaCl itself at concentrations higher than 2% inhibited partly the growth of the bacteria, they were not included in this experiment. The results of the turbidimetric measurements indicated that 2% sodium chloride increased slightly the activity of lupulon (Figure 1).

Plate-well assay showed no influence of 0.5, 1, 2, and 5% NaCl on the activity of lupulon when the salt was incorporated in the lupulon solutions. However, as illustrated in Table II slightly larger inhibition zones were produced by the same concentrations of lupulon when the salt was incorporated in the agar plates.

TABLE II
The effect of NaCl incorporated in agar plates on the activity of lupulon against *Staph. aureus*

Concentration of NaCl in agar %	Concentration of lupulon, $\mu\text{g./ml.}$			
	50	20	10	5
Average diameter of inhibition zones, mm.				
0.5	28	24	21	17.5
1.5	27.5	24	21.2	19
2.5	27.5	24.5	21.5	19.5
5.5	27.5	24.5	22	20.2

Studied with *M. tuberculosis* (H37Rv). It has also been shown by experiment, as described previously, that 2% sodium chloride increased the

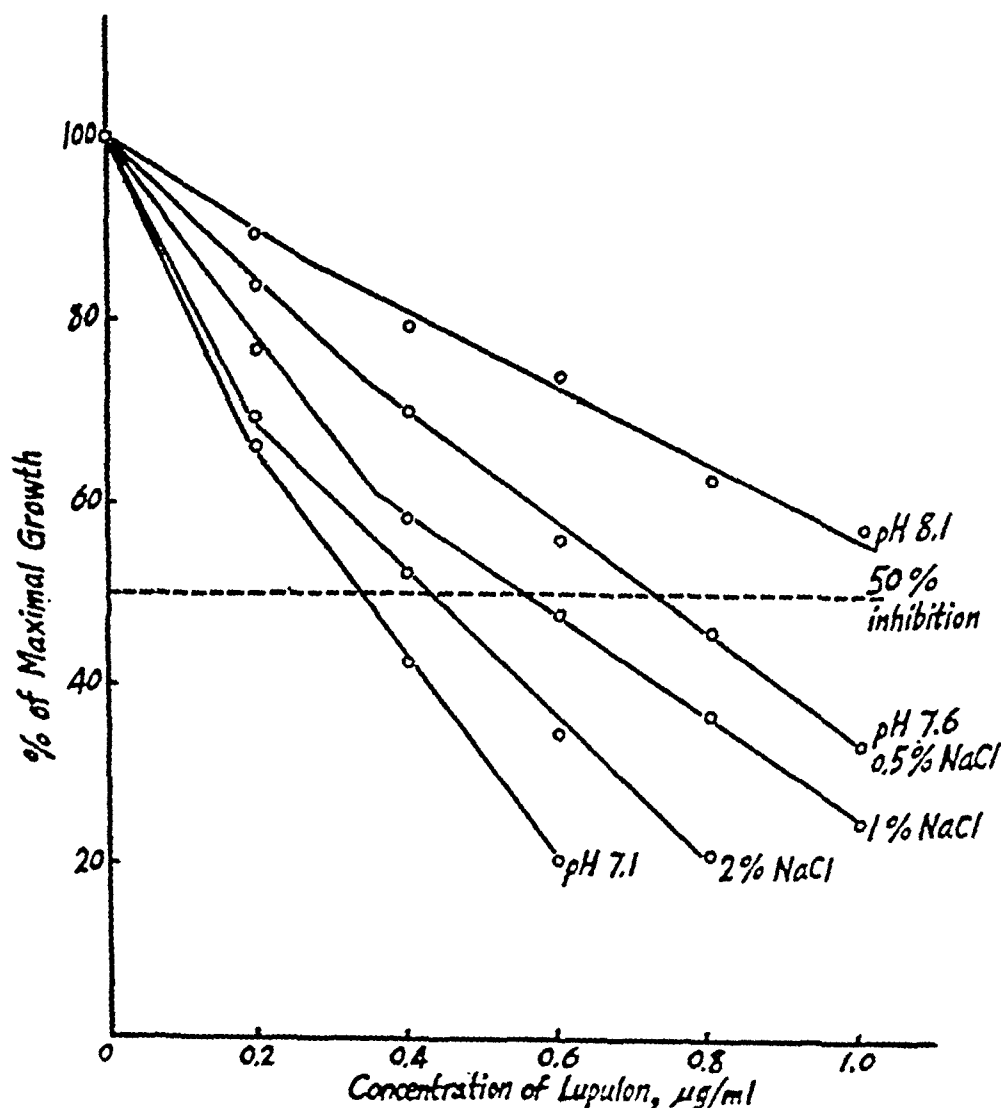


FIG. 1. EFFECT OF pH AND NaCl ON GROWTH OF *Staph. aureus* IN VARIOUS CONCENTRATIONS OF LUPULON

TABLE III

The effect of NaCl in Dubos fluid medium on the growth of *M. tuberculosis* (H37Rv) in various concentrations of lupulon

Concentration of NaCl %	Concentrations of lupulon, $\mu\text{g./ml.}$							
	40	30	25	20	15	10	5	0
0	—	—	—	++	++++	++++	++++	++++
0.5	—	—	—	+	+++	+++	+++	+++
1.0	—	—	—	+	++	++	++	++
2.0	—	—	—	—	—	++	++	++

antibiotic activity of lupulon against *M. tuberculosis* (H37Rv) (Table III).

Effect of serum

Studied with M. tuberculosis (H37Rv). Pooled human serum, Seitz-filtered and inactivated at 56° C. for 30 minutes, was added to Dubos medium at three different concentrations, namely, 5, 7, and 10%. Similarly treated horse serum was used at 10%. Since Dubos medium containing 20% of either human or horse serum became turbid and then produced sediments after 24 hours incubation at 37° C. which interfered with reading the end points, this concentration was not included in the present study. The results of repeated experiments by serial dilution method showed consistently that the growth of tubercle bacilli was completely inhibited by lupulon at a concentration of 1:40,000 or 25 $\mu\text{g./ml.}$ regardless of the presence of either serum up to 10%.

Studied with M. phlei. Table IV shows the effect of various concentrations of inactivated horse serum on the bacteriostatic level of lupulon against *M. phlei*. In the presence of 10% serum the growth was completely inhibited by 70 $\mu\text{g./ml.}$ instead of 50 $\mu\text{g./ml.}$ in its absence. There was, therefore, an apparent 30% reduction of the activity.

Studied with Staph. aureus. By the serial dilu-

tion method, using 1:1,000 dilution of 24-hour culture for inoculation, the concentration of lupulon which caused complete inhibition of growth of this bacteria in veal-glucose medium containing 10% inactivated horse serum was 12.5 $\mu\text{g./ml.}$ in contrast to 1:640,000 or 1.56 $\mu\text{g./ml.}$ in the absence of the serum. The activity of lupulon was apparently reduced to about one-tenth of its original level.

Turbidimetric and plate-well assay were then performed to give this effect a further analysis. In turbidimetry, six duplicate series of six tubes containing various amounts of lupulon were prepared as described previously in this paper. In five of such series the medium contained five different concentrations of inactivated horse serum, namely, 50, 20, 10, 5, and 2%. The sixth series served as the control without serum. The results, as shown in Figure 2, indicated that there was a reduction of the activity of lupulon to one-tenth. The growth of *Staph. aureus* was practically not inhibited by lupulon in the presence of 50% serum.

For plate assay, 0.9% NaCl was adjusted to pH 11.5. Inactivated horse serum was added to it at 2, 10, and 50%. Lupulon was diluted in these solutions at 50, 20, 10, and 5 $\mu\text{g./ml.}$ It was also diluted in 100% serum as well as the saline without serum. The inhibition zone was plotted against the logarithm of the concentrations (Figure 3). That produced by 50 $\mu\text{g./ml.}$ in 100% serum was of approximately the same size as one produced by 5 $\mu\text{g./ml.}$ in saline. The inhibitory power of lupulon in 10% serum was about 30% of that in its absence; in contrast, only one-tenth was found in the fluid medium as determined by serial dilution or turbidimetric method.

The apparently dissimilar effect of serum on the antibiotic activity of lupulon against different bacteria as well as against the same bacteria in dif-

TABLE IV

The effect of horse serum in Dubos medium on the growth of *M. phlei* in various concentrations of lupulon

Concentration of serum	Concentration of lupulon, $\mu\text{g./ml.}$									
	80	70	60	55	50	45	40	35	30	0
0	—	—	—	—	—	+	+	++	+++	++++
2	—	—	—	—	—	+	+	+++	+++	+++
5	—	—	—	+	+	+++	+++	+++	+++	+++
10	—	—	++	++	+++	+++	+++	+++	+++	+++

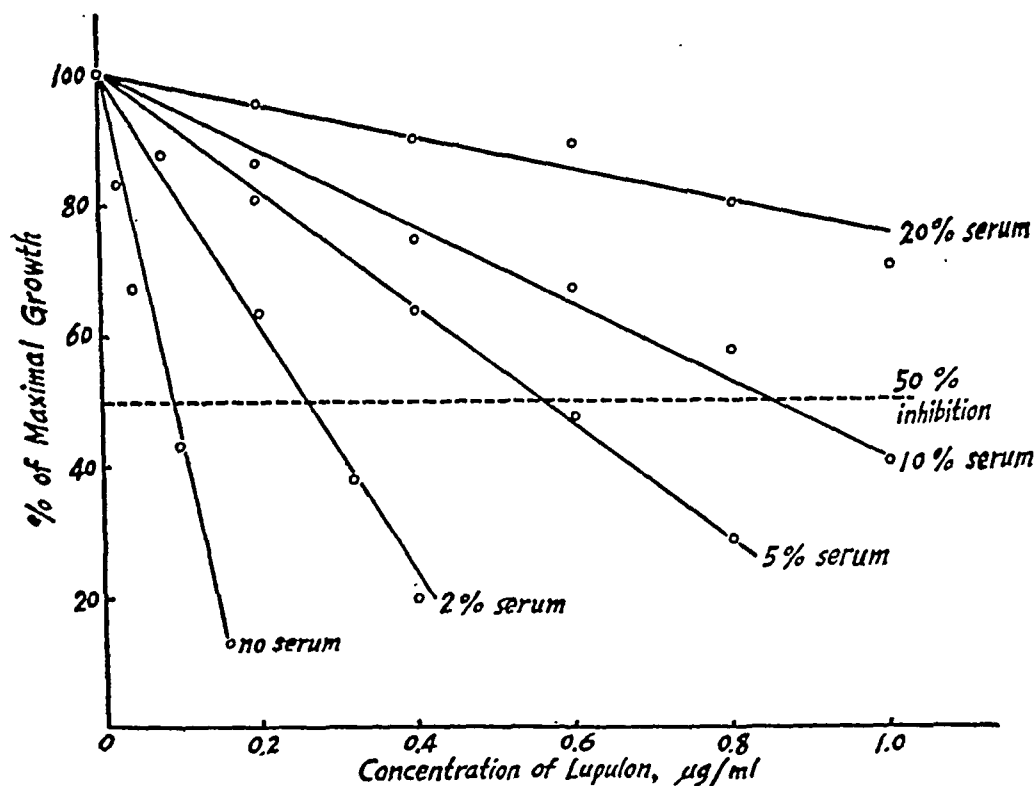


FIG. 2. EFFECT OF HORSE SERUM ON GROWTH OF *Staph. aureus* IN VARIOUS CONCENTRATIONS OF LUPULON

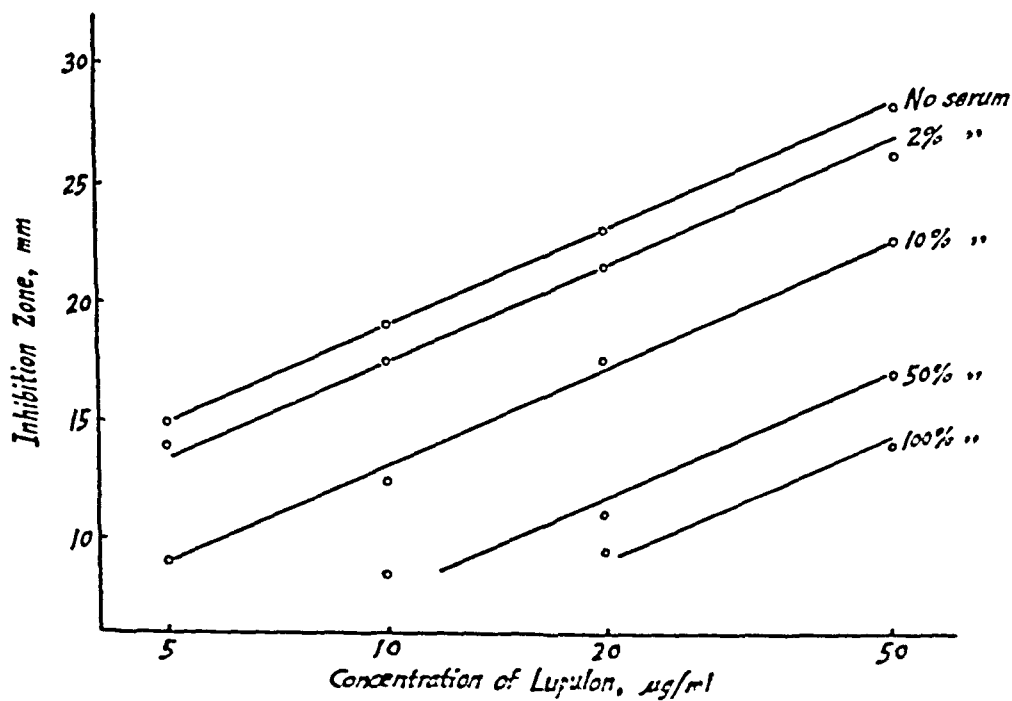


FIG. 3. EFFECT OF HORSE SERUM ON ACTIVITY OF LUPULON BY PLATE-WELL ASSAY AGAINST *Staph. aureus*

ferent media indicated that the effect was unlikely exerted on the antibiotic itself. This point was substantiated by the following experiment.

A 1,000 $\mu\text{g./ml.}$ solution of lupulon in inactivated horse serum (A) and another in alkaline water (B) were made from 1% propylene glycol solution. After 24 hours, a fresh solution of each (C and D) was prepared from the same propylene glycol solution. Thirty minutes later, equal volumes of 95% ethyl alcohol were added to all tubes, and those containing serum were centrifuged to separate the precipitates. The supernatant fluid and the alkaline-alcohol solution were diluted to 50, 20, 10, and 5 $\mu\text{g./ml.}$ of lupulon, calculated on the basis of the amount originally added to the solutions before adding ethyl alcohol. A series of alkaline solutions (E) diluted from the same propylene glycol preparation and another series of similar dilutions of the solvents only (F) served as controls. Each series of dilutions was assayed by duplicate plates. It was definitely shown (Table V) that all the activity of lupulon was retained in the supernatant fluid. It is, therefore, believed that whatever the serum contained which produced the apparent reduction in the activity of lupulon had been removed by precipita-

tion with 48% ethyl alcohol. When the same supernatant and alkaline-alcohol solutions were tested by serial dilution method the same end points were obtained.

DISCUSSION

Just how far the design of *in vitro* experiments on antibacterial agents can simulate the conditions *in vivo* is not certain. However, a study of the effect of such factors as pH, sodium chloride, and serum gives information regarding the activity of these agents before or when they act on bacteria. Such a study on lupulon has indicated that at the pH, the concentration of sodium chloride, and the presence of serum, which are normal to the body, this antibiotic remained active.

Lupulon is less soluble in water but more so in lipids at low pH. The greater partition coefficient, therefore, may serve as an explanation for its availability to the bacterial cells (10) and thus its greater antibacterial activity at lower pH. Penicillin is also more active at lower, and less at higher pH, which Abraham and Duthie (11) suggested as an explanation of the action of penicillin by competition with OH^- ions for position on the cell surface in order to produce its effect. A similar theory might be suggested also for lupulon on account of the above-mentioned facts.

Addition of sodium chloride to the media increased slightly the antibiotic activity of lupulon. In no case was it decreased, although the presence of the salt in lupulon solution assayed on plates produced no effect at all. Such minor discrepancy may be explained on the basis of a difference in the rate of diffusion as in the case of streptomycin (12).

That lupulon is not inactivated by serum has been shown by the fact that it retained activity after standing in serum solution for 24 hours. The apparent effect of serum on the antibiotic activity must be explained either by a change of sensitivity of the bacteria or by the presence of some antagonizing factors in the serum. No attempt has been made to elucidate this point. However, it is known that the apparent effect could be removed by treating solutions with 48% ethyl alcohol, whatever its causal agent may be. It is unlikely that the effect was due to growth promotion by serum, because it has been found that the same

TABLE V

The results of plate-well assay of the supernatant fluids after precipitation of proteins from lupulon solutions in horse serum with 48% ethyl alcohol

Solution assayed	Concentration of lupulon, based on the amount originally present in the solutions before adding ethyl alcohol, in $\mu\text{g./ml.}$			
	50	20	10	5
	Average diameter of inhibition zones in mm.			
Supernatant (A) after precipitation of proteins	27.5	22.5	19.5	16
(B) + equal volume of 95% alcohol	28	23	20.2	16.5
Supernatant (C) after precipitation of proteins	28	23.2	19.5	16.2
(D) + equal volume of 95% alcohol	28.2	22.7	19.7	16.2
Alkaline solutions (E) without alcohol	27.5	22.7	19.5	16
Solvent controls (F)	0	0	0	0

bacteriostatic level of lupulon against *Staph. aureus* was obtained from three different media which supported its growth to varying degrees.

The apparent activity of streptomycin varied inversely with the size of inoculum, which Berkman *et al.* (13) explained to be due to the presence of a greater number of resistant organisms in a larger inoculum. This explanation has been adopted here, with reservation, for a similar finding with lupulon.

SUMMARY

Lupulon inhibits the growth of *Staph. aureus* (FDA No. 209), *M. phlei* and *M. tuberculosis* (H37Rv) at a concentration of 1.56, 50, and 25 $\mu\text{g./ml.}$, respectively. In the case of *Staph. aureus* a higher concentration is required for a larger inoculum. It is more active at low pH. Sodium chloride at a concentration of 2% increases slightly its activity.

Its potency against *M. tuberculosis* is not affected by 10% horse or human serum. However, a 30% decrease has been observed under the same conditions for *M. phlei*. The activity against *Staph. aureus* has been estimated by serial dilution and turbidimetric methods to be reduced to one-tenth of its original level in 10% horse serum. By plate-well assay a similar reduction has been observed in 100% serum. All the activity of lupulon has been found to be retained in the supernatant fluid after precipitation of proteins from solution in horse serum with 48% ethyl alcohol. These observations argue against any inactivation of lupulon by serum.

The results of this experiment indicate that under the conditions of the pH, the concentration of sodium chloride, and the presence of serum, which

are normal to the body, lupulon should remain active.

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LUPULON AND HUMULON—ANTIBIOTIC CONSTITUENTS OF HOPS¹

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Commercial hops, the dried cones of the hop vine (*Lupulus humulus*), contain two antibacterial agents, lupulon and humulon. Methods of preparation of the compounds, their chemical structures, and their inhibitive action against Gram-positive bacteria have been known for many years; nevertheless, their antibiotic properties have been largely ignored in relation to animal infections. Their relatively high content in such an accessible source, together with the availability of simple methods for their isolation, prompted a survey of the antibiotic spectra of the substances. Lupulon and humulon were supplied to Dr. H. H. Anderson of the University of California Medical School for the determination of their activity toward *Mycobacterium tuberculosis*. The ensuing demonstration by Chin *et al.* (1) that lupulon inhibits the growth of a virulent strain of this pathogen *in vitro* and exerts a pronounced effect on experimental tuberculosis infections in mice has quickened interest in the microbiological, pharmacological, and therapeutic investigation of these agents.

The Western Regional Research Laboratory has been engaged recently in the determination of the antibiotic spectra of lupulon and humulon, development of methods by which substantial quantities of each can be readily prepared, determination of a number of their physical and chemical properties, preparation of certain derivatives, and development of methods of assay.

CHEMICAL AND PHYSICAL PROPERTIES

Structure. The formulas given in Figure 1 for lupulon and humulon had been established by 1926

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through the investigations of Wieland (2) and Wöllmer (3) on the basis of degradative reactions summarized in von Richter's Organic Chemistry (4). The agents have not been synthesized.

Solubility and stability. Both lupulon and humulon are fairly soluble in polar and non-polar organic solvents such as methanol, ethanol, petroleum ether, hexane, and iso-octane. Both are only slightly soluble in neutral or acidic aqueous solution but are readily soluble as the sodium salts.

Lupulon is moderately stable to both acid and alkali. At room temperature in the presence of air it is very labile. In the crystalline form, it may become yellow and amorphous within a few days. This change is much slower at 5° C. in air; storage for several months is accompanied by development of a characteristic odor, though color development is not marked. Lupulon crystals appear perfectly stable *in vacuo* even at 60° C. Recently, Lundin (5) has reported that oxidation of lupulon (and humulon) is promoted by daylight and by metal oxides and that the oxidation is much more rapid in petroleum ether than in alcohol. F. Stitt and G. F. Bailey of this Laboratory have found that the ultra-violet absorption spectra of very dilute solutions of lupulon in petroleum ether or in iso-octane change very rapidly on standing at room temperature, but similar solutions in methanol or water have more stable spectra.

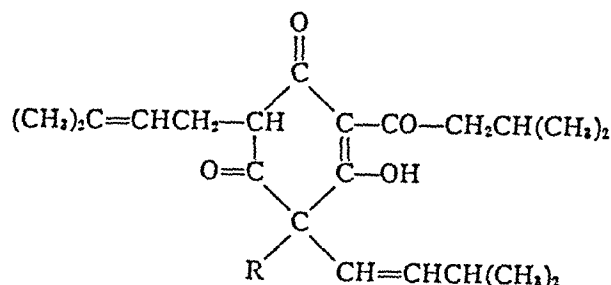


FIG. 1

Humulon: R = —OH
C₂₁H₃₂O₈, MW 362.3, m.p. 55°, [α]_D — 232°, monobasic acid.

Lupulon: R = —CH=CHCH(CH₃)₂
C₂₃H₃₄O₈, MW 414.3, m.p. 92°, optically inactive, monobasic acid.

Michener and Andersen (6) of this Laboratory found that the addition of 0.1% of ascorbic acid exerted a marked protective action on the bacteriostatic activity of lupulon steamed or autoclaved at a concentration of 4 ppm. in phosphate buffers at pH 6.5 and 8.5.

Humulon is relatively stable to acid. The lead salt is stable, but the *o*-phenylenediamine salt slowly turns brown at room temperature in air, but not *in vacuo*. In aqueous solution at neutrality, or particularly in alkaline solution, humulon is transformed by boiling to an unidentified product which is not precipitable by lead acetate but which is believed by Walker (7) to retain some antibacterial activity. Quite recently, Verzele and Govaert (8) have reported the chromatographic separation of the immediate transformation product of humulon ("isohumulon"). They state that on boiling in methanol solution, humulon is quantitatively converted to isohumulon. On boiling with aqueous alkali, they found isohumulon to be converted to humulinic acid. Humulinic acid has been prepared in this Laboratory by boiling humulon with 1 *N* NaOH (2). It proved to be antibiotically inactive.

Michener and Andersen (6) found no loss of bacteriostatic potency against *Staphylococcus aureus* when 40 ppm. of humulon in phosphate buffer at pH 6.5 or 8.5 was autoclaved. However, the presence of low concentrations of ascorbic acid extended the duration of bacteriostatic action of humulon as well as that of lupulon.

METHODS AND RESULTS

Assay

Lupulon in simple solutions may be determined by its inhibitive action on Gram-positive bacteria in turbidistic or cup-plate tests. A reduction of the bacteriostatic action of lupulon by blood serum, the mechanism of which is being investigated at this Laboratory by L. E. Sacks, prevents the use of such a method for determination of lupulon in blood and other tissues.

A tentative spectrophotometric method of analysis for the lupulon and humulon content of hop extracts has been developed by F. Stitt and G. F. Bailey of this Laboratory and will be described in a coming publication.

Isolation of hop antibiotics

Lupulon. Lupulon has been isolated on a kilogram scale in this Laboratory. The method is essentially that devised by Bungener in 1886. High-quality hops are

necessary, since lupulon is unstable and since quantitative yields are not obtained by this process.

The hops, ground with an equal weight of dry ice to reduce the stickiness of their resinous content, are extracted with petroleum ether (30–60° C.) in a column, or in countercurrent fashion in several columns. The petroleum ether extract is concentrated without delay *in vacuo* to a thin syrup, which is then placed at approximately –15° C. to crystallize. A rich extract may form a single porous cake so that the mother liquor can be drained off directly, or the crude crystals are filtered on a cold Büchner funnel. The crude crystals are then dissolved in warm petroleum ether (approx. 150 g. per l. at 40° C.), and recrystallized by chilling with stirring in a dry ice bath. The process is repeated twice, and then the lupulon is dried and dissolved in methanol (approx. 150 g. per l. at 20–25° C.). A white insoluble impurity is filtered off, and the lupulon is recrystallized by the slow addition of 1/10 volume of water and overnight storage at 0° C. The recrystallization from 90% methanol is repeated twice. High-quality hops yielded 3% of once-recrystallized and 1.5% of 6 × recrystallized lupulon, compared to the weight of the air-dry hops.

The product consists of fine white crystals, optically inactive, with theoretical C and H contents, and melting at 92–94° C. The lupulon crystals are stored *in vacuo* in the cold. Samples are distributed in evacuated ampules.

Humulon. The conventional method for the isolation of humulon by precipitation as the lead salt has been greatly simplified in this Laboratory by making the first precipitation with *o*-phenylenediamine from the crude petroleum ether extract of hops (usually from the mother liquor remaining after the crystallization of lupulon). *o*-Phenylenediamine was first used in 1916 by Wöllmer (3) for the purification of humulon lead salt. To our knowledge it has not been used previously for the direct isolation. Eighty % recovery is obtained. Free humulon can be obtained by acidification and extraction with an immiscible organic solvent such as ethyl ether.

Antibiotic spectra of humulon and lupulon

The antibiotic spectra of humulon and lupulon given in Table I have been determined in this Laboratory by the quantitative agar-streak dilution method of Waksman and Reilly (9). The antibiotics were dissolved in 1% concentration in 95% ethanol, and by means of aqueous dilutions, decreasing amounts were added to a series of 10 cm. Petri dishes, i.e., 1.0, 0.3, 0.16, 0.1, 0.03, etc., ml. per dish. The lupulon used was a preparation recrystallized three times. Humulon was tested in two forms, namely the *o*-phenylenediamine salt and humulon prepared from the above salt two hours before the test.

The test medium for bacteria and yeasts was nutrient agar (0.5% Difco peptone, 0.3% Difco meat extract, 0.5% NaCl, and 1.5% Difco agar in tap water, pH 7.0, autoclaved at 121° C. for 20 minutes). For fungi other than yeasts, potato dextrose agar was employed. Melted and cooled (45° C.) 10 ml. portions of the medium were added to plates, which were immediately and thoroughly

TABLE I

Antibiotic spectra

(Maximum dilution for complete inhibition on agar)

Type	Name	Lupulon	Humulon
Gram-Positive Bacteria	<i>Bacillus anthracis</i>	300,000	100,000
	<i>B. cereus</i> v. <i>mycoides</i>	1,000,000	100,000
	<i>B. subtilis</i>	1,000,000	50,000
	<i>Corynebacterium diphtheriae</i> gravis	100,000	10,000
	<i>Diplococcus pneumoniae</i> Type I	300,000	20,000
	<i>Micrococcus lysodeikticus</i>	300,000	60,000
	<i>M. pyogenes</i> v. <i>aureus</i>	500,000	30,000
	<i>Sarcina lutea</i>	100,000	30,000
Acid-Fast Bacteria	<i>Streptococcus faecalis</i>	500,000	30,000
	<i>Mycobacterium phlei</i>	300,000	30,000
	<i>M. tuberculosis</i> v. <i>hominis</i> (607)	100,000	10,000
Actinomycetes	<i>Streptomyces coelicolor</i>	50,000	3,000
Gram-Negative Bacteria	13 species*	<3,000	<3,000
Yeasts	4 species†	<3,000	<3,000
Fungi	7 species‡	<3,000	<3,000

* *Aerobacter aerogenes*, *Alcaligenes faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella paradyenteriae*, *Salmonella enteritidis*, *Salmonella schottmuelleri*, *Salmonella typhosa*, *Serratia marcescens*, *Shigella dysenteriae*, *Shigella paradyenteriae*.

† *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Torulopsis dattila*, *Zygosaccharomyces mandshuricus*.

‡ *Alternaria citri*, *Aspergillus niger*, *Fusarium solani* v. *pisi*, *Penicillium citrinum*, *Rhizoctonia solani*, *Rhizopus nigricans*, *Trichoderma koenigi*.

rocked to disperse the antibiotics. After about 30 minutes, cell suspensions from 24-hour-old cultures of the test organisms were streaked onto the solidified plates, each within a designated sector. Each sector received three streaks made with an L-shaped needle without recharging.

With bacteria-streaked plates, incubation was carried out at 35° C. for 18–20 hours, except for *Sarcina lutea*, the mycobacteria and the actinomycete, which were incubated for two days. Yeasts and other fungi were incubated at 30° C. for three days. After incubation, the results of growth were recorded and compared with control plates lacking the antibiotic. The inhibitory endpoint was taken as that dilution of the antibiotic which completely or nearly completely inhibited growth of the test organism. To reduce error, each spectrum was run five to ten times on different days, with replicate dilution plates each day.

Humulinic acid was found to be inactive at 0.1% for *Escherichia coli*, *Micrococcus conglomeratus*, *Micrococcus pyogenes* v. *aureus*, *S. lutea*, and *M. tuberculosis* v. *hominis*.

The inhibitive action of hop extracts towards bacteria has long been recognized and turned to practical use in brewing and in preservation of unpasteurized beer. In 1937 Shimwell (10) pointed out that Gram-negative bacteria would

grow as readily in hopped as in unhopped beer worts, whereas Gram-positive bacteria grew not at all or very poorly in hopped wort. Walker and Parker (11) presented data which showed that 0.8 ppm. of humulon or 0.2 ppm. of lupulon give 50% inhibition of acid production by a strain of *Lactobacillus bulgaricus* inoculated into grain wort at pH 4.5. Hansen (12) found pure humulon and lupulon to exert no inhibitive action at 50 ppm. on several species of *Saccharomyces*, on *E. coli*, *Eberthella typhosa*, and *Salmonella paratyphus* A and B. Both *Staph. aureus* and *Bacillus mesentericus* were inhibited at 12 and 2 ppm. of humulon and lupulon, respectively, while germination of spores of *Bacillus subtilis* was inhibited by 4 and 0.5 ppm., respectively. Michener, Snell, and Jansen (13) found that humulon and lupulon possess a low order of antibiotic activity against a panel of plant pathogenic fungi. Yeasts were largely unaffected.

The tuberculostatic action of lupulon was first noted by Chin *et al.* (1) and confirmed by Salle *et al.* (14) and by ourselves (see above). Chin found lupulon active against the H37Rv strain of *M. tuberculosis* at 1:40,000 in Dubos' medium, while Salle found the same strain inhibited by 1:200,000 in Long's medium and in Proskauer and Beck's medium, and by 1:90,000 in Dubos' medium. Humulon was much less active, and was not tested further.

The possibility of the development of lupulon-fast strains of Gram-positive and acid-fast bacteria has not been investigated as yet, but the brewing literature contains references to the "acclimatization" of Gram-positive bacteria to beer wort.

Serum has been found by Salle *et al.* (14), by Chin *et al.* (15), and by ourselves to reduce the bacteriostatic action of lupulon *in vitro*. In serial dilution tests with *Staph. aureus* and *M. conglomeratus* in broth containing 0.7 or 7% of serum, we found approximately 50 µg. of lupulon to be inactivated per ml. of undiluted serum. In cup-plate tests with *Mycobacterium phlei* and *B. subtilis*, 9 ppm. of lupulon in 10% serum gave zones equivalent to those given by 2 ppm. of lupulon in water.

Although inactivation by serum *in vitro* might by present concepts eliminate an antibiotic from further consideration for internal and other applications, such an effect does not prove a lack of therapeutic

tic value. In fact, the positive influence of lupulon administered orally or intramuscularly in oil on mouse tuberculosis as described elsewhere (1) suggests that otherwise promising tuberculostatic drugs if discarded solely because of inactivation by serum *in vitro* should be reevaluated by animal infection tests.

SUMMARY

The isolation, chemistry, assay, and antibiotic spectra of lupulon and humulon have been briefly discussed. A Bureau of Agricultural and Industrial Chemistry Circular of Information entitled "Antibacterial Agents from Hops," in which are summarized the findings of past and present studies of these substances, is now available for distribution.

The Western Regional Research Laboratory is preparing lupulon and humulon in amounts which will allow for distribution on a limited scale to parties interested in their evaluation.

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THE OCCURRENCE OF ANTIBACTERIAL SUBSTANCES ACTIVE AGAINST *MYCOBACTERIUM TUBERCULOSIS* IN SEED PLANTS¹

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For many centuries green plants have been used for the treatment of various diseases. Tuberculosis is no exception, and numerous plants and plant products have, at one time or another, been reported to be of value in the treatment of this disease.

During the past few years a number of investigators have surveyed seed plants for sources of antibacterial activity (1-10). In most of these studies a Gram-positive organism, *Staphylococcus aureus* or *Bacillus subtilis*, and a Gram-negative organism, *Escherichia coli* or *Salmonella typhosa*, were used for detecting antibacterial properties. In some cases the active materials or purified preparations derived from them were then tested against a variety of microorganisms.

In this exploratory survey a strain of *Mycobacterium tuberculosis* was used for assay because of the assumption that some plants might contain substances specifically antibacterial for the tubercle bacillus. These would be missed in any assay using other test organisms. To obtain more information on the antibacterial properties of the extracts, however, most of them were also tested for their effect on *Staph. aureus* and *E. coli*.

Early in the study it was found that some plants contained substances active against the tubercle bacillus but that all or much of the activity was lost in the presence of whole blood. In subsequent tests the active plant extracts were also assayed in media to which blood had been added.

METHODS

Crude plant materials were used for the preliminary screening. The plants were either used in a fresh state immediately after collection, or dried and extracted at a later date. Whenever possible the use of entire plants for

preparation of representative extracts was avoided. In most instances, either leaves or stems or roots or, if the plant was in the stage of flowering, inflorescences were extracted separately. Sometimes different organs had to be combined because of insufficient amounts of material.

The plants tested were green plants, the majority belonging to the subdivision Angiospermae. The system of classification used follows the synopsis of the plant kingdom, published in L. H. Bailey's Standard Cyclopedia of Horticulture (11) and based on "Die Natürlichen Pflanzenfamilien" by Engler and Prantl.

The preparation of the plant material to be tested was essentially the one described by Lucas *et al.* (12). A Waring Blendor was the main tool for the disintegration of the plant parts. However, in the present study extractions with boiling water and with ethanol were made in addition to the customary cold-water extraction. The ethanol extraction followed the technique of the cold-water extraction with the exception that paper was used instead of cheesecloth for the filtration of the macerates. The hot extraction was performed by immersion of the plant materials in boiling water for five to ten minutes, the time depending on the properties of the tissues. All cold-water extracts and any ethanol extracts found to be contaminated were sterilized by passing them through a Seitz filter, boiled preparations by autoclaving.

The extracts were tested for antibacterial properties by making serial dilutions in liquefied glycerol beef extract agar, pH 7.0. The agar was allowed to harden in a slanting position, and after solidification the tubes were inoculated with an 18-to-40-day-old culture of *M. tuberculosis*, strain H37, grown on Petragnani's medium. To prevent evaporation during the long incubation period screw-capped tubes were used for making the cultures. After six weeks of incubation at 37° C. the cultures were examined. If they were inhibited the highest dilution in which no growth occurred was recorded. The active extracts were then retested on glycerol beef extract agar to which 10% whole sheep blood had been added.

With the alcoholic extracts allowance had to be made for the inhibitory effect of the alcohol. This solvent was found to inhibit the tubercle bacillus at a dilution of 1:40 and therefore only extracts in which there was no growth at a dilution higher than this were considered active.

The antibacterial activity of the extracts against *Staph. aureus* (F.D.A. strain 209P) and *E. coli* (SA436) was determined by making serial dilutions in nutrient broth (F.D.A. formula). The broth was inoculated with 1.0 ml. of an 18-to-24-hour broth culture of the organism per 100 ml. of medium just before making the dilutions.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

Results were read after incubation for 24 and 48 hours at 37° C.

It was found that ethanol inhibited both *Staph. aureus* and *E. coli* at a 1:8 dilution. Consequently only extracts showing an inhibition greater than this dilution were recorded as being antibacterial.

RESULTS

The results of the survey are presented in Table I. They are interesting in many respects. Approximately half the number of species with antibacterial activity against *M. tuberculosis* showed it only against this organism; the others were active also against other organisms. These findings cannot be considered as final since the concentration of the active principles is an unknown factor. It is possible that in some instances materials used merely appeared specific because the antibacterial principle was present in a concentration too low to affect the other or-

TABLE I—Continued

Plant name	Plant part	Result
<i>Juniperus communis</i>	Fr	—
<i>Digitaria sanguinalis</i>	E	—
<i>Panicum miliaceum</i>	Fl and L	—
	St and R	—
<i>Phleum pratense</i>	E	—
<i>Billbergia pyramidalis</i>	Fl and L	—
<i>Agave attenuata</i>	L	—
<i>Aloe africana</i>	L	—
<i>A. arborescens</i>	L	—
<i>A. aristata</i>	L	—
<i>A. barbadensis</i>	L	—
<i>A. Brunnhaleri</i>	L	—
<i>A. cameronii</i>	L	—
<i>A. candelabrum</i>	L	—
<i>A. chinensis</i>	L	A + +, B + + +, C + + + Sa, Ec
<i>A. commutata</i>	L	—
<i>A. distans</i>	L	—
<i>A. Eru</i>	L	—
<i>A. ferox</i>	L	—
<i>A. grandidentata</i>	L	—
<i>A. humilis</i>	L	—
<i>A. Marlothii</i>	L	—
<i>A. microstigma</i>	L	—
<i>A. mitriformis</i>	L	—
<i>A. nobilis</i>	L	—
<i>A. plicatilis</i>	L	—
<i>A. robusta</i>	L	—
<i>A. saponaria</i>	L	—
<i>A. spinosissima</i>	L	—
<i>A. striata</i>	L	—
<i>A. succotrina</i>	L	A +, B +, C + + +
<i>A. Thornecroftii</i>	L	—
<i>A. variegata</i>	L	—
<i>A. virens</i>	L	—
<i>A. xerina</i>	L	—
<i>Colchicum autumnale</i>	R	—
<i>Gasteraloe prethamensis</i>	L	—
<i>Gasteria acinacifolia</i>	L	—
<i>G. carinata</i>	L	—
<i>G. excelsa</i>	L	—
<i>G. maculata</i>	L	—
<i>G. obtusa</i>	L	—
<i>G. picta</i>	L	—
<i>G. punctata</i>	L	—
<i>Smilax officinalis</i>	R	—
<i>S. ornata</i>	R	—

Plant name	Plant part	Result
<i>Tulbaghia violacea</i>	E	A +, Ec
<i>Urginea maritima</i>	L	—
<i>Amaryllis Belladonna</i>	Fl and L	—
	St and R	—
<i>Iris germanica</i>	L	—
<i>Myrica asplenifolia</i>	L	B + +, C + + +
<i>Betula alba</i>	Bark	—
<i>Humulus lupulus</i>	Fl	B + +, C + + +, Sa, Ec
<i>Parietaria officinalis</i>	L	—
<i>Urtica urens</i>	L and St	—
<i>Polygonum Persicaria</i>	L	Sa
<i>Rheum officinale</i>	St	—
	R	A + +, B + +, C + + +
<i>Spinacia oleracea</i>	E	—
<i>Amaranthus hypochondriacus</i>	L, St and R	—
<i>A. retroflexus</i>	E	—, Sa, Ec
<i>Phytolacca decandra</i>	R and Fr	—
<i>Portulaca grandiflora</i>	E	—
<i>Anemone hepatica</i>	L	—
<i>Hydrastis canadensis</i>	R	A +, Sa
<i>Paeonia officinalis</i>	L	—
	St	—, Sa, Ec
<i>Berberis asiatica</i>	Bark	—, Sa, Ec
<i>Caulophyllum thalictroides</i>	R	—
<i>Jeffersonia diphylla</i>	R	—
<i>Mahonia trifoliata</i>	E	—
<i>Podophyllum peltatum</i>	R	—
<i>Anamirta Cocculus</i>	Fr	—
<i>Menispermum canadense</i>	R	B + +
	F	—
<i>Sanguinaria canadensis</i>	R	A +, B + +, C + + +, Sa
<i>Brassica nigra</i>	Fl and L	—
	St and R	—
<i>Crassula arborescens</i>	L	—
<i>Kalanchoe flammula</i>	L	—
<i>Sedum pachyphyllum</i>	L	—
<i>Sempervivum tectorum</i>	Fl, L and R	—
<i>Hamamelis virginiana</i>	Bark	—
<i>Prunus domestica</i>	Fr	—, Sa, Ec
<i>Pyrus malus</i>	Pollen	—
<i>Sanguisorba minor</i>	E	—
<i>Sorbus Americana</i>	Fr	—
<i>S. aucuparia</i>	Bark	—
<i>Baptisia tinctoria</i>	R	—
<i>Pisum sativum</i>	L and St	—
<i>Trifolium hybridum</i>	E	B +
<i>Vicia sativa</i>	E	—
<i>Geranium maculatum</i>	Fl, L and St	—
<i>Ruta graveolens</i>	E	—
<i>Euphorbia heterophylla</i>	Fl and L	—
	St and R	—
<i>E. variegata</i>	Fl and L	A +, B +
	St and R	—
<i>Ilex paraguariensis</i>	L	—
<i>Eonymus atropurpurea</i>	Bark	—
<i>Acer platanoides</i>	L and St	—
<i>Impatiens Balsamina</i>	Fl and L	B +, Sa
	St and R	—
<i>Zizyphus Jujuba</i>	Fr	—
<i>Tilia euchlora</i>	Fl	—
<i>T. platyphyllos</i>	—	—
<i>Althaea officinalis</i>	Fl, L and R	—
<i>Hypericum calycinum</i>	L	C + + +
<i>Epiphyllum truncatum</i>	L	—
<i>Eucalyptus globulus</i>	L	C + + +
<i>Myrtus communis</i>	L and St	—
<i>Aralia nudicaulis</i>	R	—
<i>Panax quinquefolium</i>	R	—
<i>Archangelica officinalis</i>	R	—
<i>Daucus Carota</i>	E	—
<i>Eryngium amethystinum</i>	L and St	—
<i>E. aquaticum</i>	R	—
<i>Foeniculum vulgare</i>	L and St	—
<i>Lernicium officinale</i>	L and St	—
<i>Ardostaphylos Uva-Ursi</i>	L	—
<i>Gaultheria procumbens</i>	L and St	—
<i>Yuccinia angustifolia</i>	Fr	—, Sa, Ec
<i>V. corymbotum</i>	E	A +, B +, Sa
<i>Gentiana lutea</i>	R	—, Sa
<i>Asclepias Cernuifolia</i>	E	—
<i>Phlox Drummondii</i>	Fl and L	—
	St	—
<i>Eriodictyon platyneuron</i>	L	C + + +, Sa
<i>Erythronium officinale</i>	L and St	—
<i>Palmetto officinalis</i>	Fl and L	—
	St and R	—, Sa
<i>Lippia citrifolia</i>	L and St	—
<i>Celastrus Flumen</i>	L	—, Sa
	St and R	—
<i>Lecythis Cordata</i>	L and St	—
<i>Menispermum canadense</i>	L and St	—

TABLE I—Continued

Plant name	Plant part	Result
<i>M. sylvestris</i>	Fl and L	—, Sa
	St	—
<i>Monarda punctata</i>	E	—, Sa
<i>Salvia officinalis</i>	L	B +, C + + +, Sa
	St and R	—, Sa
<i>S. splendens</i>	Fl and L	—
	St	—
<i>Capsicum annuum</i>	Se and Fr	—
<i>Hyoscyamus niger</i>	E	—, Sa
<i>Lycopersicum esculentum</i>	Fl, L and St	—
<i>Petunia hybrida</i>	E	—
<i>Verbascum Thapsus</i>	E	—
<i>Veronica officinalis</i>	L and St	—, Sa
<i>Plantago major</i>	L	—
<i>Lonicera tatarica</i>	Fr	—, Ec
<i>Sambucus niger</i>	Fr	—
<i>Valeriana officinalis</i>	R	—
<i>Dipsacus sylvestris</i>	Se	—
<i>Anthemis nobilis</i>	Fl	—
<i>Arctium minus</i>	R	A +
<i>Arnica montana</i>	Fl and R	—
<i>Centaurea americana</i>	Fl and L	B +, Sa
	St and R	—
	L and St	—
<i>C. jacea</i>	E	A +, Sa, Ec
<i>Chrysanthemum parthenium</i>	E	—
<i>Cichorium Intybus</i>	Se	—
<i>Cirsium arvense</i>	L	A + +
<i>C. lanceolatum</i>	St	—
<i>Cosmos bipinnatus</i>	Fl and L	—
	St and R	—
<i>Dahlia variabilis</i>	Fl	A +, B +, Sa
<i>Erigeron canadensis</i>	Fl and L	A +, B +
	St and R	—
<i>Helianthus tuberosus</i>	Fl and L	—
	St and R	—, Sa, Ec
<i>Matricaria Chamomilla</i>	Fl	A +, B +, Ec
<i>Onopordon Acanthium</i>	E	—
<i>Silphium perfoliatum</i>	Fl	—
	L	A +
<i>Silybum Marianum</i>	Fl and L	—
	St and R	—
<i>Tagetes erecta</i>	Fl, L and St	—
<i>Tanacetum vulgare</i>	L and St	—
<i>Zinnia elegans</i>	Fl, L, St	—

Activity against *M. tuberculosis* is indicated by +, ++, or +++, according to potency of extracts. A, B, or C preceding these signs indicates cold water, boiling water, or ethanol extract respectively. Lack of activity is signified by —. Sa and Ec denote activity against *Staph. aureus* and *E. coli*.

Plant parts are designated as follows: Entire plant, E; flowers, Fl; leaves, L; stems, St; roots, R; seeds, Se, and fruits Fr.

ganisms. Nevertheless, by testing the plant extracts against the customary organisms only, half of the materials with activity against the tubercle bacillus would have been overlooked.

Some of the extracts antibacterial for *M. tuberculosis* were tested using for assay the rapidly growing acid-fast strain 607. Only three out of 20 extracts were found to be active against this acid-fast strain.

The materials in which activity against the tubercle bacillus was observed were derived from 27 species, which belonged to 16 families. Eight species belonged to the family Compositae, three to Liliaceae, two to Euphorbiaceae, two to Labiatae. Six of the materials with exclusive activity against *M. tuberculosis* were from Compositae and two from Euphorbiaceae.

Plant leaves were carriers of active principles in most cases. Nine of the 27 plants showed activity in the leaf extracts. In five cases it was found in the extract of leaves and flowers combined while flowers alone showed activity only in three cases. In five plants the activity was found in the roots, and four of the extracts prepared from entire plants inhibited growth of the tubercle bacillus. No activity was recorded in seeds and fruits. Stems and roots combined showed activity in one case. This plant, *Coleus Blumei*, is the only one where extracts of different parts showed specific activities, the leaf extract being active against *Staph. aureus*, the stems and roots against the tubercle bacillus. There were two other cases, one in the same family, where the apparent specificity might be explained by the greater sensitivity of *Staph. aureus* to the active principle. However, in both cases the possibility of the presence of more than one antibacterial principle could not be ruled out.

Only one of the 35 antibacterial plant samples did not show a loss of activity in the presence of whole blood. The other extracts lost all or part of their antibacterial properties in contact with it.

This report is the first in a series involving the investigation of seed plants for principles antagonistic to the tubercle bacillus. Testing of crude materials is being continued. Some of the plant materials, which in the exploratory phase inhibited the growth of *M. tuberculosis*, are under further study.

As Kavanagh (13) very aptly expressed it, "compounds active against more than a few common bacteria must be found, if the potentialities of antibiotic substances as specific inhibitors of microorganisms are to be realized fully. This can be done only by using the organism to be inhibited as the test object in the survey."

SUMMARY

The antibacterial activity against *M. tuberculosis*, strain H37, of 211 plant samples from 161 species belonging to 53 families of seed plants was determined by serial dilution tests. Each sample was extracted separately with cold water, boiling water and ethanol. Twenty-seven species were active, 13 of them specifically against *M. tuberculosis*, while not interfering with the growth of

Staph. aureus and *E. coli*. The activity was found in various parts of the plants, mostly in the leaves.

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A CRYSTALLINE ANTIFUNGAL AGENT, MYCOSUBTILIN, ISOLATED FROM SUBTILIN BROTH¹

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The presence of an antifungal agent in the broth of subtilin cultures has been reported (1). It is not unusual in cultures of aerobic spore formers to find several different antibiotics present. Often, these have been related polypeptides, which have different biological spectra of antibiotic action (gramicidin, tyrocidine, etc.).

This paper reports the isolation and properties of a fungistatic substance from the cells of a culture of *Bacillus subtilis* 370, originally obtained from the Western Regional Research Laboratory of the United States Department of Agriculture, where it was used for studies on subtilin (2). Under certain cultural conditions, fungistatic activity is produced which is not due to subtilin. The standard assay organism for the fungistatic agent, *Trichophyton* sp. MF 301 was not inhibited by 200 μ gm. of subtilin per ml. of agar. The antifungal agent has tentatively been named Mycosubtilin.

METHODS AND RESULTS

Conditions for production

Submerged aerated cultures of *B. subtilis* 370 were grown in 250 ml. flasks containing 50 ml. of 20% beet molasses with 0.8% $(\text{NH}_4)_2\text{HPO}_4$ and 0.005% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ on a shaker rotating at 220 RPM (3). The production of subtilin proceeds at optimum rate at a temperature of 34° C., but a temperature of 25° C. was found more suitable for the fungistatic substance. Yields were better at this lower temperature and filtration and extractions were conducted with greater ease. Fungistatic activity was produced in five days under the foregoing conditions.

Fermented cultures produced in a semi-synthetic medium containing 0.5% yeast extract² gave broth activity equal to that in cultures prepared with beet molasses

medium. Broth samples were usually mixed with three volumes of ethanol to aid extraction of the antibiotic and to sterilize the culture for assay. The agar streak-plate method was used, with *Trichophyton* sp. MF 301 as the test organism, with two-fold dilution levels.

Extraction

The harvested broth was adjusted to about pH 2.5 with concentrated hydrochloric acid to precipitate the cellular material, which was separated by centrifugation. The first extraction of the moist cell solids was made overnight with 95% ethanol, which resulted in a final concentration of approximately 70% ethanol. Subsequent extractions of the cells were made overnight with 70% ethanol solutions. The solids were separated from the ethanol suspensions by filtration. The active fraction was precipitated quantitatively by the addition of two volumes of water and was separated by centrifugation. The solids were successively extracted with 95% ethanol. Material of increased purity was obtained with each successive extract. The ethanol was evaporated to dryness and all of the resulting solids dissolved in a very small quantity of pyridine. White crystalline material was separated during a period of several hours at 10° C. following addition of 10 volumes of water. The crystals were washed with water and recrystallized from 70% ethanol.

It was later observed that the solids from the 70% ethanol extracts of the cells could be directly dissolved in pyridine, thus eliminating the 95% EtOH stages, with better yields. Several recrystallizations were necessary to obtain material which showed a constant melting point and analysis.

The results in Table I represent a typical extraction from a culture produced in beet molasses medium, which had a moist cell volume of 15%.

Properties

The white crystals are practically insoluble in reagents, with the exception of pyridine and 70% ethanol. They are very soluble in the former but show only limited solubility in warm 70% EtOH. The antifungal agent crystallized readily from the latter upon cooling. The crystals are soluble in

justed to pH 6.8-6.9 with NH_4OH and brought to one liter volume. The formula for the semi-synthetic medium and details of the procedures used at the Western Regional Research Laboratory for production of subtilin were disclosed to us by personal communication.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² The semi-synthetic medium contained sucrose, 100 gm.; citric acid, 11.7 gm.; Na_2SO_4 , 4 gm.; yeast extract, 5 gm.; $(\text{NH}_4)_2\text{HPO}_4$, 4.2 gm.; KCl , 0.76 gm.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.42 gm.; ZnCl_2 , 0.0104 gm.; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.0245 gm.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0181 gm., dissolved in water, ad-

TABLE I
 Isolation data for antifungal agent

	Volume	Solids dry wt.	Activity dilution	Total activity
	ml.	mgm./ml.	units/mgm.	
Original whole broth	1080	79.3	1.9	162,000
<i>Cell extraction</i>				
First (95% EtOH)	103	36.4	19.2	72,000
Second (70% EtOH)	96	21.5	46.5	96,000
Third (70% EtOH)	100	12.9	54.3	70,000
<i>Extraction of solids</i>				
First (95% EtOH)	17	28.6	35.0	17,000
Second (95% EtOH)	19	9.9	101	19,000
Third (95% EtOH)	19	2.4	208	9,500
Fourth (95% EtOH)	15	1.0	400	6,000
Fifth (95% EtOH)	19	0.6	416	4,750
Crystallization from pyridine	—	88.9 (total)	700	62,000

dilute cold NaOH but insoluble in dilute cold HCl and cold NaHCO_3 .

No precipitate was obtained with 2,4-dinitrophenylhydrazine. There was no reduction of Fehling solution nor color development with ferric chloride solution. Millon's reagent produced a temporary pink coloration and nitration with conc. HNO_3 formed a pale yellow pigment. Acid hydrolyzates gave a strongly positive ninhydrin reaction.

Following recrystallization three times from 70% ethyl alcohol, the crystals melted with decomposition at $256^\circ\text{--}257^\circ\text{C}$. (corr.) on a micro block.³ The crystals gave an analysis of C—55.31, 55.12; H—7.61, 7.33; N—15.15, 15.18. Sulfur was not present. The ultra-violet absorption spectrum of alcohol-water solution consisted of a band at 2770 \AA with an $E_{1\%}^{1\text{cm}} = 14.6$. Potentiometric titration showed an apparent combining weight by alkali titration of 1980. General insolubility made the compound unsuitable for molecular weight determination.

Ten mg. of crystalline mycosubtilin was hydrolyzed in a sealed tube with 3 ml. of 6 *N* HCl at 120°C . for 16 hours. Paper strip chromatography with a phenol-water system, followed by develop-

³ We are indebted to Dr. E. F. Rogers for helpful suggestions and criticisms concerning the chemical characterization. Mr. R. N. Boos performed the elemental analysis and Dr. Charles Rosenblum the UV spectrum and potentiometric titration.

ment with ninhydrin, yielded four distinct zones, with observed R_f values presented in Table II, and an indistinct zone at R_f 0.97, close to the advancing solvent boundary. Amino acids which have characteristic R_f values in the region of the zones observed with mycosubtilin are listed in Table II. Aspartic acid, tyrosine and proline were found to be present by microbiological assay. The natural isomers of the other amino acids listed, with the exception of norvaline and norleucine for which no assays were available, were not found to be present by microbiological assay in concentrations exceeding 5%. The microbiological assay for aspartic acid and alanine is not specific for the natural form. Therefore, it is not certain which isomer of aspartic acid was present and it was demonstrated that the D form of alanine was not present. The tyrosine content is adequate to account for the ultra-violet absorption band described above. It is interesting to note that tyrosine has not been found in subtilin, the other amino acid-containing antibiotic produced by *B. subtilis* 370.

Aspartic acid accounts for nearly one-half the molecule and one-third of the nitrogen. An ad-

 TABLE II
 Amino acid composition of mycosubtilin and subtilin HCl hydrolyzate

Mycosubtilin			Subtilin composition†
Observed R_f	Literature values*	Observed microbiological assay	
.19	Aspartic acid (.19)	45%	Aspartic acid Lanthionine
.34	Glutamic acid (.32) Serine (.37)	— —	L-glutamic acid Glycine L-lysine
.62	Alanine (.63) Tyrosine (.63) Arginine (.66)	— 5.4% —	Alanine
	Valine (.82) Norvaline (.84)	— —	Tryptophane L-valine
.85	Isoleucine (.88) Leucine (.88) Norleucine (.82) Phenylalanine (.93) Proline (.93)	— — — — 4.2%	L-isoleucine L-leucine L-phenylalanine L-proline
.97			

* Pratt, J. J. and Auchair, J. L. Science, 1944, 102, 213.
 † Lewis, J. C. and Allen, G. A. C. S. Abstracts, 1944.

TABLE III
Antibiotic activity of mycosubtilin against yeasts

	Complete inhibition μgm./ml.
<i>Candida guilliermondii</i> , 488	N.A. at 20
<i>Debaryomyces gruelzii</i> , 4144	N.A. at 20
<i>Schwannomyces occidentalis</i>	N.A. at 20
<i>Zygopichia californica</i>	N.A. at 20
<i>Mycoderma valida</i>	7.5
<i>Saccharomyces carlsbergensis</i> , 9080	7.5
<i>Rhodotorula rubra</i>	Ca. 5.0
<i>Sporobolomyces roseus</i>	5.0
<i>Torula cremoris</i>	5.0
<i>Dipodascus uninucleatus</i>	3.75
<i>Hansenula anomala</i> , 4104	3.75
<i>Torulopsis delbrückii</i>	3.75

(N.A. indicating no inhibition)

TABLE IV
Fungistatic activity of mycosubtilin

	Complete inhibition μgm./ml.
<i>Aspergillus niger</i>	N.A. at 20
<i>Trichoderma</i> sp.	N.A. at 20
<i>Mucor flavus</i>	N.A. at 20
<i>Rhizopus javanicus</i> takeda	N.A. at 20
<i>Microsporium lanosum</i> *	N.A. at 20
<i>Trichophyton mentagrophytes</i> *	10.0
<i>Fusarium moniliforme</i>	10.0
<i>Nematospore coryli</i>	7.5
<i>Penicillium notatum</i>	7.5
<i>Chaetomium bostrychodes</i>	7.5
<i>Microsporium audouinii</i> *	5.0
<i>Achorion schoenleinii</i> *	5.0
<i>Cryptococcus neoformans</i> *	5.0
<i>Epidermophyton inguinale</i> *	5.0
<i>Sclerotinia fructicola</i>	5.0
<i>Ustilago zae</i>	2.5
<i>Trichophyton</i> sp.	1.5

(N.A. indicating no inhibition)

* We are indebted to Dr. Morris Solotorovsky for the assay of these particular pathogenic fungi and for toxicity determinations.

ditional one-third of the nitrogen, 5.1% N, was liberated as NH_3 by acid hydrolysis. The speculation arises that aspartic acid is present in the molecule as the amide.

The substance is heat stable, resisting autoclaving in agar for 15 minutes at 120°C . and 15 lbs. pressure.

A concentration of 0.0015 mgm./ml. of agar inhibited the growth of the test organism *Trichophyton* sp. (MF 301) by the streak-plate method on yeast extract dextrose agar. However, the addition of 10% horse serum incorporated in the agar completely neutralized the activity.

Antibiotic spectrum

Micrococcus lysodeikticus was inhibited by 0.001 mgm./ml. of nutrient agar by the streak-plate method. But 0.016 mgm./ml. failed to inhibit *Staphylococcus aureus* (Smith), *Streptococcus pyogenes* C-223, *Streptococcus viridans*, *E.*

coli, *Eberthella typhosa*, *Klebsiella pneumoniae*, *Hemophilus pertussis*, *Corynebacterium diphtheriae* (gravis), *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* 607, *Salmonella aertrycke*, *Salmonella paratyphi* A, *Salmonella schottmuelleri*, *Alcaligenes faecalis*, and *Bacillus megatherium*.

The yeast spectrum, Table III, and the fungus spectrum, Table IV, were streaked on yeast extract dextrose agar. The results indicate the smallest amount of crystalline material required to cause complete inhibition. Although mycosubtilin is insoluble in water, it does not precipitate when a 10 mgm./ml. solution in hot 70% alcohol is added to liquid agar to make a concentration of 1 mgm./ml.

Toxicity

Toxicity was determined by the subcutaneous injection of mycosubtilin into white Swiss mice weighing approximately 20 gms. each. For injection into mice, a solution containing 2 mgm. of agent per ml. was prepared by mixing an alcoholic solution of the crystalline material with 20% aqueous gelatin. Deaths were observed following single doses of 1 or 0.5 mgm., but single doses of 0.25 mgm. or less were tolerated.

It was impossible to demonstrate the agent in the blood of injected animals because of the neutralizing effect of blood serum on the antifungal activity.

SUMMARY

A fungistatic substance has been isolated from the cells of *B. subtilis*, the subtilin producing microorganism. A method of extraction has been suggested by which crystalline material has been obtained. The white crystals, having a melting point of $256^\circ\text{--}257^\circ\text{C}$. with decomposition, yield amino acids following acid hydrolysis. The new antibiotic has been named mycosubtilin.

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THE ANTIFUNGAL ACTIVITY OF ANTIBIOTIC XG¹

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Lewis, Hopper, and Shultz, in 1946 (1), reported the existence of an antimicrobial agent in culture filtrates of an organism closely related to *Bacillus subtilis*, and demonstrated that this substance possesses a remarkable antifungal activity against *Trichophyton gypsum* and certain other pathogenic fungi, as well as against certain of the Gram-positive bacteria. During the following year, Lewis and Hopper undertook to investigate methods of producing this antibiotic on a small scale, and succeeded in recovering a sufficient quantity to permit preliminary study of its biological and chemical properties as well as its therapeutic efficacy in fungous infections in man. These early studies of Lewis and his associates suggested that Antibiotic XG may be a highly effective chemotherapeutic agent in the treatment of fungous infections in man.

The present report will discuss certain of the chemical and biological properties of this new antifungal agent.²

NATURE OF ANTIBIOTIC

Antibiotic XG is produced by the growth of an unidentified organism closely related to *B. subtilis*. The antibiotic may be absorbed from fermentation liquor onto carbon at neutral pH and may be eluted with aqueous acetone. It is soluble in methanol and ethanol. It is insoluble in ether, chloroform, ethyl acetate, and dry acetone.

Antibiotic XG is stable in broth when heated at 90° C. for two hours at pH 3.0; it may be autoclaved at 15 pounds pressure, for at least 10 minutes, at pH 7.0 without significant loss in potency. In wet acetone, it is stable at room temperature

for at least eight months, and in anhydrous alcohol, at a concentration of 3,500 units per cc., it is stable for at least two months.

Antibiotic XG is an amphoteric polypeptide which gels on standing in water. The gel form is biologically inactive but can be reactivated by dissolving in anhydrous solvents. The polypeptide may be hydrolyzed and on paper chromatography shows the presence of several different amino acids.

METHODS AND RESULTS

Standardization

The activity of Antibiotic XG is measured in terms of *T. gypsum* dilution units, one unit being the least amount of antibiotic which will inhibit the growth of a standard strain of *T. gypsum* under controlled conditions. The master standard used for all biological assays of Antibiotic XG has an activity of 70 *T. gypsum* dilution units per mg.³ Preparations having potencies as high as 250 units per mg. have been obtained.

In vitro activity

Methods. The sensitivities of a wide variety of microorganisms to Antibiotic XG were determined in the following manner. Except when otherwise specified, a beef infusion broth (2) buffered at pH 7.8 was used throughout. It was recognized that other mediums, such as Sabouraud's agar, are more commonly used for growth of the pathogenic fungi. It was felt advisable to use the beef infusion broth, however, in view of the fact that it appeared suitable for the growth of both the pathogenic bacteria and fungi.

In the case of all bacteria tested and in the case of *Monilia albicans*, 15-16 hour broth cultures were used except when otherwise indicated. In the case of other fungi than *M. albicans*, suspensions of six to eight day cultures on Sabouraud's agar were used. In all instances, bacterial cultures were diluted in broth to a constant density immediately prior to use. A density allowing 82-86% transmission on a Photovolt Lumetron No. 40 was arbitrarily chosen as standard. Suspensions of the fungous cultures were diluted to approximately the same

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² The preparations of Antibiotic XG used in this study were prepared in the Biochemical and Chemical Research and Development Departments of Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

³ We are indebted to Dr. B. A. S. Van der Vliet for all assay data reported herein.

TABLE I
The Antimicrobial Action of Antibiotic XG
Medium: Nutrient Broth

Organisms	No serum Sensitivity: units/cc.	1% Rabbit serum	5% Rabbit serum
<i>Sporotrichum schenkii</i>	11.2	11.2	5.6
<i>Microsporium canis</i>	0.7	0.7	1.4
<i>Trichophyton rubrum</i>	11.2	2.8	1.4
<i>Cryptococcus neoformans</i>	1.4	2.8	5.6
<i>Trichophyton gypsum</i>	2.8	1.4	0.7
<i>Epidermophyton floccosum</i>	2.8	1.4	0.7
<i>Microsporium audouinii</i>	11.2	1.4	1.4
<i>Rhodotorula</i>	1.4	1.4	5.6
<i>Monilia albicans</i>	1.0*	90.0	90.0

* On prolonged incubation sensitivity of this one organism frequently decreased to 90–100 units per cc.

Sensitivity tests were carried out in broth and were read after two days' incubation. Sensitivity was accepted as the least amount of Antibiotic XG causing complete inhibition of growth.

density but clumping of the organisms prevented exact standardization by means of the Photovolt Lumetron.

A graded serial dilution technic was used in all tests. To each 1.8 cc. of serially diluted antibiotic, 0.2 cc. of a 10^{-4} dilution of bacterial culture or 0.2 cc. of an undiluted suspension of fungous culture was added. Incubation was carried out at 37° C. The amount of growth was recorded at 24 and 48 hours in the case of bacteria and at two and five days in the case of fungi. The sensitivity of an organism was accepted as the least amount of Antibiotic XG which would cause complete inhibition of growth, as indicated by absence of gross turbidity. It was recognized, however, that this did not necessarily indicate a bactericidal level.

Results. Antibiotic XG is a highly efficient antifungal agent, active against all strains of pathogenic fungi tested. As shown in Table I, it is active in the presence of serum as well as in its

TABLE II
The antibacterial action of Antibiotic XG

Organism	Sensitivity: units/cc.	
	24 hours	48 hours
<i>Streptococcus hemo.</i>	4.0	64
<i>Staphylococcus aureus</i>	>315	>315
<i>Diplococcus pneumoniae</i>	2.0	31.5
<i>Escherichia coli</i> (BW41)	>315	>315
<i>E. coli</i> (W57)	>315	>315
<i>Escherichia typhosa</i>	>315	>315
<i>Salmonella dys.</i>	>315	>315
<i>S. paratyphosa</i>	>315	>315
<i>Acetabacter aerogenes</i>	>315	>315
<i>Proteus vulgaris</i>	>315	>315
<i>Klebsiella pneumoniae</i>	>315	>315
<i>Pseudomonas pyocyaneus</i>	>315	>315
<i>Pseudomonas leucovorax</i>	< 1.7	>315

absence. In general, sensitivities varied little with time of incubation. In the case of *M. albicans*, growth was rapid in the medium used and in this instance sensitivity varied markedly depending upon the length of the incubation period.

The sensitivity of 13 strains of microorganisms, belonging to 10 different bacterial species, is shown in Table II. A temporary bacteriostatic effect was demonstrable against *Streptococcus hemolyticus* (Strain C203 Mv) and against *Diplococcus pneumoniae* (Strain I/230). All other bacteria tested were completely resistant to the action of Antibiotic XG, as prepared in our laboratories.

Mechanism of action

The early studies on the sensitivity of various microorganisms to Antibiotic XG indicated that this antimicrobial agent is strongly fungistatic in nature, and that its action may be affected markedly by changes in the number of organisms present, the concentration of the agent, the temperature of incubation, as well as other factors. To study the effect of Antibiotic XG on certain of the sporulating or slowly-growing pathogenic fungi, by means of growth curves such as are often used in the study of the action of antimicrobial agents on bacteria, seemed impractical. *M. albicans* and a *Rhodotorula*, however, have lent themselves readily to this type of microbiological test.

Methods. Preparations of Antibiotic XG, ranging in potency from 65–100 units per mg., were used throughout these experiments. Growth curves were carried out in the usual manner, as described in a previous communication (3). In certain experiments, all factors were held constant with the exception of the number of organisms; in other experiments, the concentration of drug or the temperature of incubation was varied. In all instances, the number of organisms per cc. at any given time during the incubation period was determined by the pour plate technic.

Effect of concentration of Antibiotic XG on growth of certain pathogenic fungi. The effect of varying concentrations of Antibiotic XG on the growth of *M. albicans*, at 37° C. and at 26° C., is shown in Figures 1 and 2. At 37° C., there was no growth of *M. albicans* when the initial number of organisms was in excess of 1,000,000 organisms per cc. Concentrations of 500–1,000 units of Antibiotic XG were sufficient, however, to produce an apparent fungicidal effect when the initial number

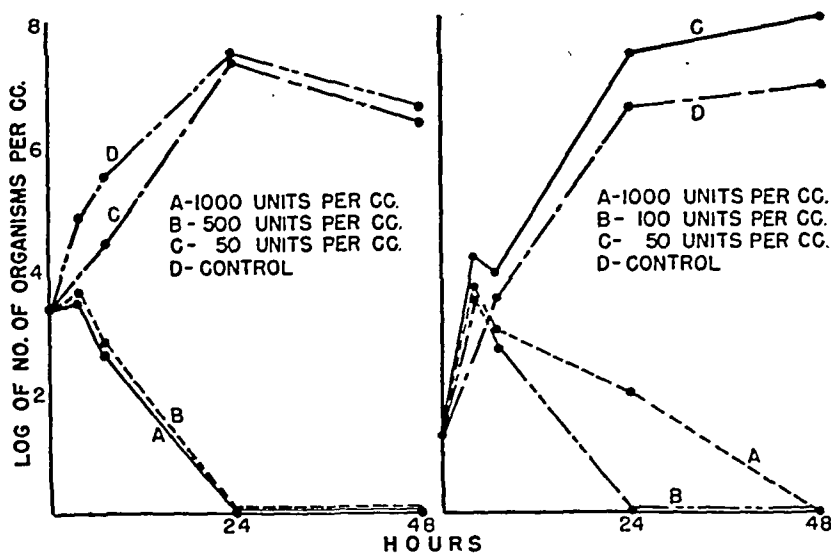


FIG. 1. THE EFFECT OF VARYING CONCENTRATIONS OF ANTIBIOTIC XG ON GROWTH OF *Monilia albicans* (37° C.)

of organisms was in the vicinity of 10,000 organisms per cc. With this number of organisms, 50 units per cc. produced no effect on the growth of the organism. With smaller numbers of organisms, 100-1,000 units produced routinely an in-

itial stimulation of growth followed by a gradual decrease in the number of organisms present. As with larger numbers of organisms, 50 units per cc. was totally ineffective.

The data presented indicate that growth of *M.*

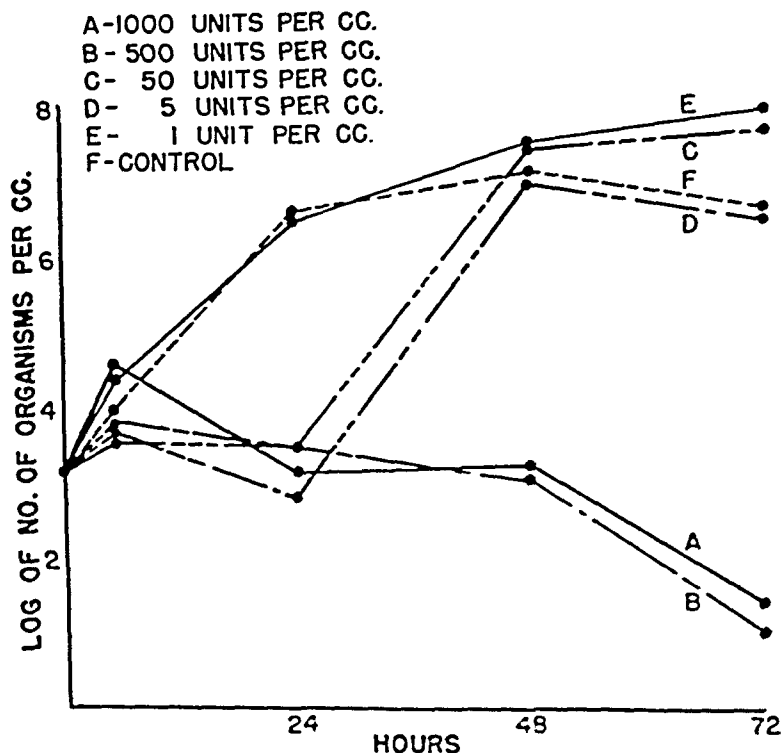


FIG. 2. THE EFFECT OF VARYING CONCENTRATIONS ON ANTIBIOTIC XG ON GROWTH OF *Monilia albicans* (26° C.)

albicans in the medium used is more rapid at 37° C. than at 26° C. In like manner, the action of Antibiotic XG is more complete at 37° C. than at 26° C. No decrease in the number of organisms present was apparent in any instance unless there was also evidence of multiplication.

The effect of varying concentrations of Antibiotic XG on a more sensitive organism than *M. albicans*, namely a *Rhodotorula*, is shown in Figure 3. In this instance, the fungistatic action of Antibiotic XG was apparent even in the presence of an initial concentration of 1,000,000 organisms per cc. Ten to 100 units per cc. was sufficient to produce a gradual decrease in the microbial count. Two units per cc. caused an inhibition of multiplication only, with no actual decrease in number of organisms, while 0.5 unit per cc. allowed full growth. It should be noted that in contrast to *M. albicans*, the *Rhodotorula* produced some multiplication in the absence of Antibiotic XG, under the conditions of this experiment.

Effect of varying concentrations of organisms on the action of Antibiotic XG. In the presence of 1,000 units of antibiotic per cc. of medium, Antibiotic XG exerted a fungicidal action on *M. albicans*, provided incubation was carried out at 37°

C., and provided the initial number of organisms was less than 10,000 per cc. If incubation was carried out at 26° C., a fungistatic effect was apparent, again providing the initial number of organisms was less than 10,000 per cc. With larger numbers of organisms, no fungistatic or fungicidal action on the part of the antibiotic could be demonstrated against *M. albicans*. (See Figure 4.)

Similar results were obtained with the *Rhodotorula*, an organism much more sensitive to the action of Antibiotic XG. In the presence of 0.5–1.0 unit per cc. rapid growth occurred regardless of the number of organisms present. In the presence of 2.0 units per cc., the number of organisms decreased rapidly to zero provided the initial number present was less than 10,000 per cc. It should be mentioned, however, that this does not represent a true fungicidal effect. No growth was observed in pour plates examined after 48 hours of incubation. After incubation for four or five days, however, small numbers of minute colonies appeared, indicating that the antibiotic had served only to suppress the metabolism of the organism. (See Figure 5.)

The effect of hydration on the action of Antibiotic XG. It has been stated previously that An-

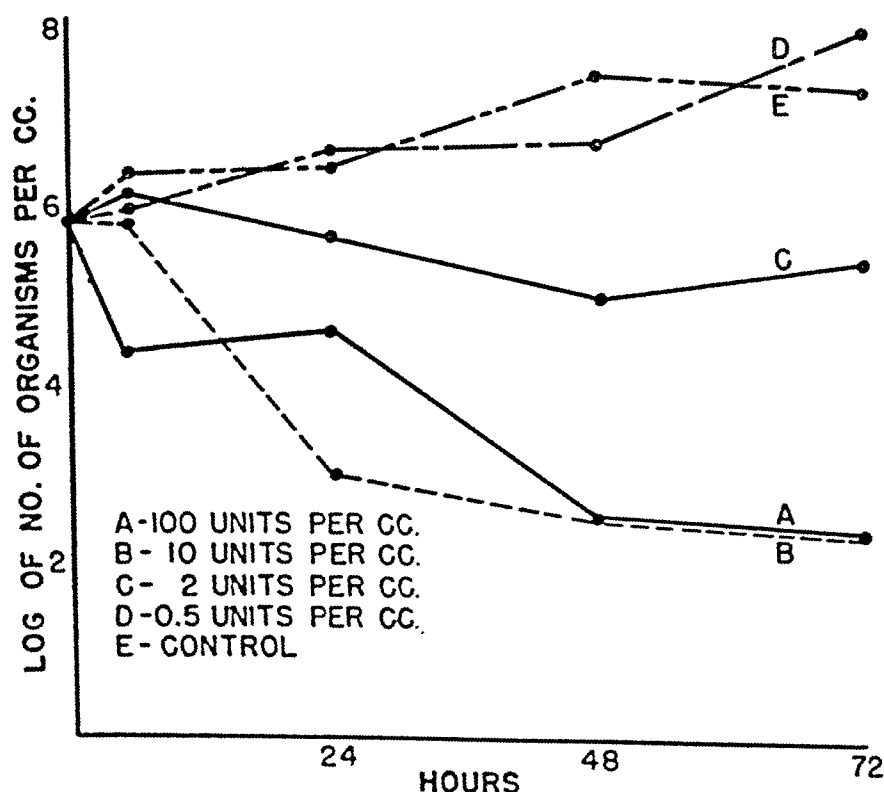


FIG. 3. THE EFFECT OF VARYING CONCENTRATIONS OF ANTIBIOTIC XG ON GROWTH OF *Rhodotorula* (26° C.)

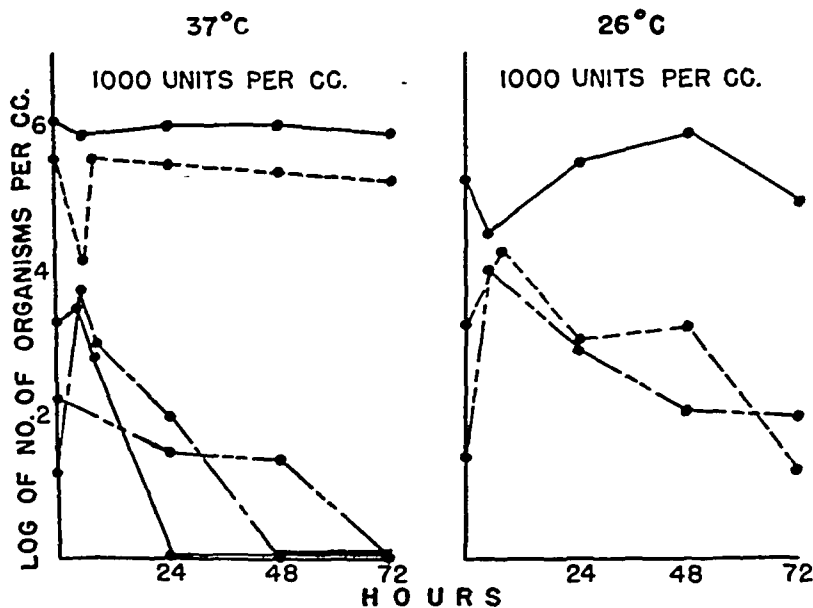


FIG. 4. THE EFFECT OF VARYING CONCENTRATIONS OF ORGANISMS ON THE ACTION OF ANTIBIOTIC XG (*Monilia albicans*)

antibiotic XG on standing in water forms a hydrated gel which is biologically inactive. The difference in the activity of the dehydrated active form of the antibiotic and the hydrated gel form was readily demonstrable, as shown in Figure 6. Five hundred units of the dehydrated form per cc., although not completely inhibitory under the conditions of the experiment, were more active than

1,000 units of the hydrated or gel form per cc. The unitage of the latter form was calculated on the basis of the potency per mg. of the same preparation prior to hydration.

Toxicity

Antibiotic XG in its present degree of purity is a strongly hemolytic compound *in vitro*. The tox-

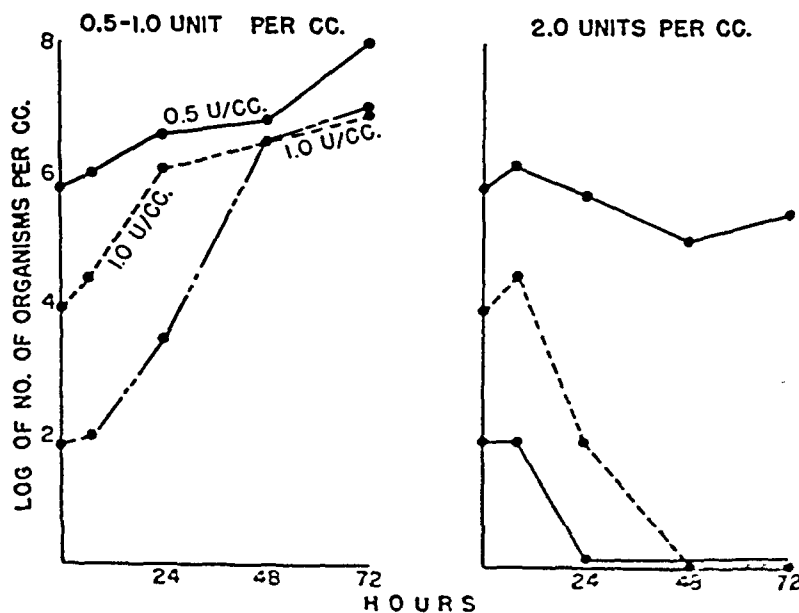


FIG. 5. THE EFFECT OF VARYING NUMBERS OF ORGANISMS ON ANTIFUNGAL ACTION OF ANTIBIOTIC XG (*Exedynals*—26° C.)

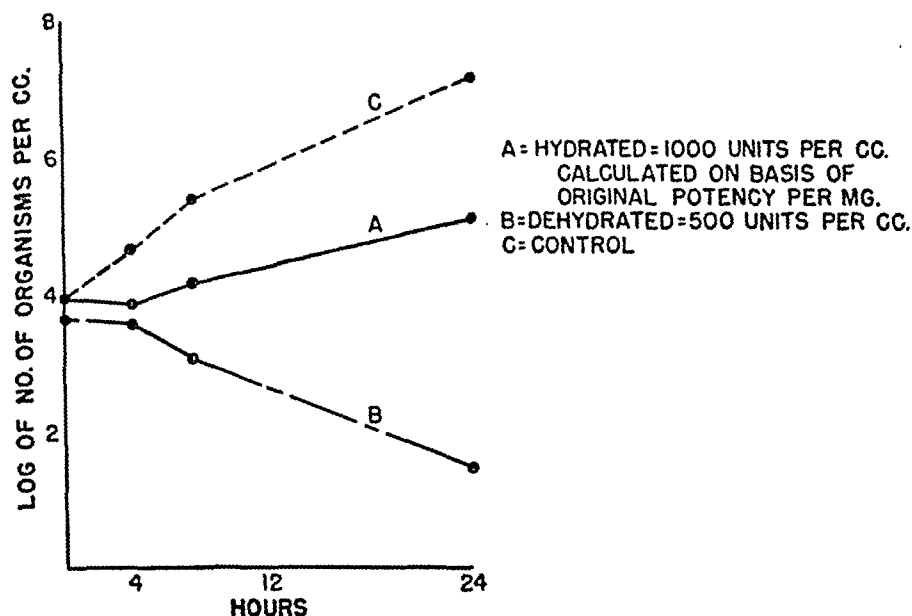


FIG. 6. THE EFFECT OF HYDRATION ON ACTION OF ANTIBIOTIC XG
(*Monilia albicans*—37° C.)

icity of this antimicrobial agent is less than would be anticipated, nevertheless, on the basis of its hemolytic action.

The acute intravenous toxicity in mice has varied from lot to lot. The LD₀ and LD₅₀ of aqueous solutions of the most highly purified preparation tested (110 units per mg.) was 5,500 and 9,900 units respectively per kg. mouse. The same preparation when dissolved in saline showed an acute intravenous LD₀ and LD₅₀ of 11,000 and 16,500 units respectively per kg. mouse.

Repeated subcutaneous injections of this preparation have indicated that aqueous solutions in dosages of at least 4,100 units per kg. mouse may be administered twice daily for a period of at least five days with no toxic manifestations. (See Table III.)

Rats receiving a single subcutaneous injection of 30,000 units of a less highly purified lot per kg. body weight showed no signs of proteinuria or other toxicity.

Rabbits receiving dosages of 5,000–10,000 units per kg. body weight intravenously, in aqueous solution, in most instances have died promptly after injection. The mortality may be lowered slightly by the use of saline in place of water as diluent for injection. Intravenous dosages of 500–1,000 units per kg. body weight generally cause no reactions. By the intramuscular or oral routes, rabbits tolerate readily 2,000–5,000 units per kg. body weight in either aqueous or saline solutions.

Whether or not the antibiotic actually is absorbed following injection in these animals remains to be determined. Preliminary studies on

TABLE III
Cumulative Toxicity in Mice
Antibiotic XG

Daily Dosage Regimen Units/kg. mouse	No. of Mice	Per Cent Survived
4100 q.d.	10	100
4100 bid	10	100
5500 q.d.	10	100
5500 bid	10	60

All animals were treated by the subcutaneous route for five days or until time of death.

the absorption and excretion of Antibiotic XG have suggested that the drug must be absorbed to some extent; the concentrations of antibiotic detected in the serum, however, have been low and erratic. That the antibiotic is bound to or inactivated by plasma protein seems unlikely in view of the fact that the *in vitro* sensitivities of a wide variety of microorganisms to this antibiotic are not altered by the presence of serum. It seems probable that failure to detect larger quantities of Antibiotic XG in serum following parenteral administration is due to the fact that proper assay techniques have not been developed to permit detection of the full amount of antibiotic present in the body fluids.

Chemotherapeutic action

It is difficult to evaluate adequately the chemotherapeutic potentialities of an antimicrobial agent such as Antibiotic XG⁴ by animal experimentation. In general, the fungous infections which occur in man are not readily reproduced in animals. Studies on the chemotherapeutic action of an agent such as this, therefore, can be carried out most satisfactorily directly in man.

The hemolytic action of Antibiotic XG until recently has discouraged its administration by parenteral routes. The fungous group of infections, however, represents one group of diseases in which topical therapy would take care of the great majority of cases. For this reason, Antibiotic XG is of interest despite its hemolytic action. Preliminary clinical studies on the topical and parenteral use of this agent are in progress and indicate (5)

⁴ Antibiotic XG possesses certain properties in common with Bacillomycin as described by Landy *et al.* (4). Comparative studies on the chemical nature of these two compounds and on their biological action are in progress.

that this antibiotic offers considerable promise in the topical treatment of *Monilia* and *T. gypsum* infections as well as in the treatment of a variety of other fungous diseases.

CONCLUSIONS

Antibiotic XG is a highly active antimicrobial agent which exerts a remarkable inhibitory effect on the growth of many of the pathogenic fungi. It is both fungistatic and fungicidal, depending on the experimental conditions used. Its activity is influenced by the number of organisms present, the rate of growth of the organisms, the concentration of antibiotic, and the temperature of incubation. In its present degree of purity, it is hemolytic in nature. Preliminary studies indicate a relatively low degree of toxicity, however, and suggest that with purification and further understanding of the mechanism of its action, it may be possible to administer it parenterally. In its present form, Antibiotic XG offers great promise as a chemotherapeutic agent for the treatment of a group of infections in which local therapy usually is adequate.

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NEOMYCIN—PRODUCTION AND ANTIBIOTIC PROPERTIES^{1, 2, 3}

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ANTIBIOTIC SURVEYS

During the last 10 years, a large number of antibiotics which are active against Gram-negative and Gram-positive bacteria, mycobacteria, rickettsiae, and certain of the larger viruses were isolated (1) from various species and strains of the genus *Streptomyces*. This served to focus attention on the actinomycetes as potential producers of antimicrobial agents that might possess promising chemotherapeutic properties. The fact that nearly 20 to 50% of these organisms possess antimicrobial activities served to heighten this interest. Numerous surveys have been conducted. Particular attention has been paid to the formation and isolation of antibiotics that would possess the following characteristics: (a) High activity against Gram-negative bacteria and mycobacteria; (b) antibiotic action against streptomycin-resistant bacteria; (c) low toxicity to animals; (d) other desirable properties, such as activity against rickettsiae, viruses, tumors and phages.

In our own laboratories, large numbers of actinomycetes were isolated from various natural substrates and tested for their antimicrobial activities. The agar-cross-streak method, frequently supplemented by other procedures, was commonly used for screening purposes. Those cultures that proved to be most active were selected and grown in liquid media. Only a small number of these were found capable of giving rise to active antibiotics. The most promising were selected for further studies. For example, of some 300 freshly isolated cultures tested by the above method, only 10 exhibited activities that justified further study.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² Paper in the Journal Series, New Jersey Experiment Station, Rutgers University—The State University of New Jersey, Department of Microbiology.

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All these cultures proved to be highly active against mycobacteria.

Only few of the cultures, however, that gave good activity by the agar-streak method yielded filtrates which possessed corresponding potency. This may be due to a variety of factors, such as the formation by a single organism of more than one antibiotic or the production of different antibiotics under different conditions of culture. One of these cultures proved to be highly promising and was selected for more detailed investigations. This culture was entered into the Collection as No. 3535. The nature of its antimicrobial spectrum, as compared to corresponding spectra of known antibiotic-producing organisms and measured by the agar-cross-streak method, is shown in Table I. The spectrum of 3535 was found to be quite distinct from those of the streptomycin-producing *Streptomyces griseus* and streptothricin-producing *Streptomyces lavendulae*. The streptomycin-resistant strain of *Escherichia coli* and the Bodenheimer organism were found to be sensitive to the antibiotic activity of 3535; further, the antibiotic

TABLE I

Antibiotic spectrum of culture No. 3535 as compared to spectra of streptothricin-producing (*S. lavendulae*) and streptomycin-producing (*S. griseus*) organisms
Agar-cross-streak method. Zone of inhibition, in mm.

Test organisms*	Culture No. 3535	<i>S. griseus</i>	<i>S. lavendulae</i>
		3463	3516
<i>Escherichia coli</i> SS	18	Active	20
<i>E. coli</i> RS	25	None	Active
<i>E. coli</i> DS	NG	GG	NG
<i>Bacillus subtilis</i>	21	Active	22
<i>B. mycoides</i>	20	Active	3
<i>B. cereus</i>	18	Active	5
<i>Staphylococcus aureus</i>	19	Active	19
Bodenheimer's culture	19	None	Active
<i>Pseudomonas aeruginosa</i>	3	LA	LA
<i>Proteus vulgaris</i>	22	Active	Active
<i>Mycobacterium</i> 607	24	25	25
<i>M. avium</i>	20	20	23
<i>M. ranae</i>	15	25	23
<i>M. phlei</i>	28	27	26

* SS = streptomycin-sensitive; RS = streptomycin-resistant; DS = streptomycin-dependent; NG = no growth; GG = good growth; LA = limited activity.

TABLE II

Effect of CaCO₃ upon the production of antibiotic 3535

Incubation, days	No CaCO ₃		CaCO ₃ , 1%	
	pH	u./ml.	pH	u./ml.
2	5.6	25	6.8	35
3	5.8	47	6.7	88
5	6.1	68	7.8	172
6	7.7	156	8.0	200

substance produced by 3535 cannot replace streptomycin in making possible the growth of the streptomycin-dependent strain of *E. coli*. The activity of 3535 upon *Bacillus mycoides* and *Bacillus cereus* serves to differentiate this antibiotic from streptothricin. The various mycobacteria were found to be sensitive alike to the antibiotic produced by 3535 and to the other two antibiotics.

METHODS

Formation and isolation of antibiotic 3535

The next steps in the study of the new antibiotic comprised the development of suitable media for its maximum production. Fortunately, media previously found to be best for the production of streptomycin proved suitable. A medium containing 5 gm. peptone, 5 gm. meat extract, 5 gm. glucose, 5 gm. NaCl, 1,000 ml. tap water gave good results. Various modifications were later introduced. The reaction of the medium tended to become acid during the early stage of growth; this favored early lysis of the

TABLE III

Influence of composition of medium on the production of antibiotic 3535

Constituents of medium, gm./liter	N	N ₁	N ₂	N ₃	No. 3
Meat extract	5		5	5	5
Soya peptone	10	10	20	10	
Bacto peptone					5
NaCl	5	5	5	5	5
Glucose	10	10	10	20	10
Tap water	1000	1000	1000	1000	1000

Medium	Incubation, days					
	3		4		6	
N	pH*	u./ml.†	pH	u./ml.	pH	u./ml.
N ₁	8.3	100	8.4	240	8.7	240
N ₂	8.2	<10	8.2	10	8.4	75
N ₃	8.6	150	8.6	240	9.0	150
N ₄	7.4	15	7.8	240	7.9	100
No. 3	8.4	<10	8.5	10	8.8	10

* Initial pH of medium 6.5-6.8.

† u./ml. = *E. coli* dilution units, as measured by agar streak dilution method.

culture. This effect was overcome by addition of CaCO₃, as brought out in Table II. This could also be accomplished by increasing the peptone content of the medium to 1 or even 2%, particularly when vegetable peptones, such as soya peptone, were used, or by reducing the sugar content. If sufficient peptone were used, the glucose content could also be increased, with a delaying but favorable effect on the production of the antibiotic. The results of a typical experiment are given in Table III. The addition of a small amount of zinc (1 mg. to 10 mg. ZnSO₄·7H₂O per liter) was later found to exert a favorable

TABLE IV

Influence of composition of improved media upon the production of antibiotic 3535

Dilution units per 1 ml. of medium; cup readings made against a neomycin standard

Nature of medium	pH	<i>E. coli</i>	<i>B. mycoides</i>	<i>S. aureus</i>	<i>B. subtilis</i>	Cup readings u./ml.
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three days incubation

N ₂ *	7.8	50	240	150	750	35
N ₁ †	7.6	50	300	300	>1,000	24
N ₃	7.5	25	100	75	500	28
N ₄	7.4	150	>1,000	>1,000	>1,000	154
N _{7a}	6.5	<30	<30	<30	240	<5

four days incubation

N ₂	8.3	50	300	240	1,000	56
N ₄	7.8	150	300	300	2,400	85
N ₃	8.3	75	240	300	>1,000	68
N ₄	8.0	500	2,400	1,500	>10,000	316
N _{7a}	6.0	<10	30	10	>100	<5

six days incubation

N ₂	8.7	100	300	240	1,000	56
N ₄	8.7	200	1,000	900	>3,000	128
N ₃	8.7	90	240	240	1,000	74
N ₄	8.7	900	7,500	3,000	>10,000	407
N _{7a}	8.4	30	150	100	>300	24

* N₂ = Soya peptone—2%, glucose—1%, NaCl—0.5%, meat extract—0.5%, tap H₂O—1000 ml.† N₁ = N₂ minus meat extract; N₃ = N₂ minus NaCl; N₄ = N₂ + 10 mg./liter ZnSO₄·7H₂O; N_{7a} = N₂ + 2% glucose.

effect, yielding culture filtrates with an activity of 500 to 1,000 u./ml., as shown in Table IV. Larger amounts of zinc proved to be injurious, however. Meat extract could be replaced by yeast extract. Glucose could be replaced by starch in shaken cultures. When distilled water was used, no activity was obtained unless zinc was added.

Aeration proved to be an important factor in the production of the antibiotic, the amount of air required being apparently less than for the production of streptomycin and streptothricin. The reduced aeration could be accomplished by increasing the volume of the medium in the

shaken flasks or by adding a small amount of agar to the medium. A higher temperature (35° C.) was found to be more favorable for rapid formation of neomycin than a lower temperature (26–28° C.); however, a higher level of activity was reached upon prolonged incubation at 28° C.

In measuring the potency or concentration of the new antibiotic, the agar plate dilution method was first used, since it not only allowed determination of the total activity but also tended to establish the antibacterial spectrum of the culture. The agar-diffusion or cup method, which has proved so successful in measuring the potency of other antibiotics, required certain modifications and a well defined standard. This is brought out in the following summary of the effect of different concentrations of antibiotic 3535 upon its antibacterial activity, as measured in terms of streptomycin units:

Concentration of antibiotic 3535 mg./ml.	Activity, as measured in terms of streptomycin u./mg.
1.00	10
0.10	18
0.01	94

When measured by the agar-streak dilution method, the same preparations gave 30 *E. coli* units per 1 mg.

The antibiotic was removed from the broth by procedures similar to those previously found to be effective in the isolation of streptothricin (2) and streptomycin (3).

RESULTS

Antimicrobial properties of antibiotic 3535

The first crude preparations gave an antibacterial spectrum which was quite characteristic of this antibiotic. This is brought out in Table V. One would expect from the results of the agar-cross-streak tests that the new antibiotic would be active alike upon the streptomycin-sensitive and the streptomycin-resistant organisms, as well as upon the streptothricin-sensitive and -resistant organisms. This was actually found to be the case. The high activity of the new antibiotic upon the various mycobacteria proved to be especially interesting. When the sensitivity of the human pathogenic culture of *Mycobacterium tuberculosis* H37Rv and of the streptomycin-resistant strain H37RvR was determined by turbidimetric procedures in the Dubos Tween medium, both cultures were found to be equally sensitive. The new antibiotic was similar to streptomycin in its lack of activity upon fungi.

The differences in the antimicrobial spectra of streptomycin and the new antibiotic, combined with certain chemical differences between the two

TABLE V
Comparative antibacterial spectra of
neomycin and streptomycin
Amounts required to inhibit growth of organism
in 1 ml. of culture

Organism	Neomycin* u./ml.	Streptomycin† μg./ml.
<i>Aerobacter aerogenes</i>	0.625	0.5–2.5
<i>Bacillus anthracis</i>	0.156	0.375
<i>B. mycoides</i>	0.1–0.5	0.1–3.8
<i>B. subtilis</i>	0.02–0.1	0.12–1.0
<i>Brucella abortus</i>	1.25–5.0	0.5–3.75
<i>B. melitensis</i>	0.625–2.5	0.5
<i>B. suis</i>	0.312–2.5	0.5
<i>Clostridium perfringens</i>	>10.0	>104
<i>Corynebacterium diphtheriae</i>	0.156	0.375–3.75
<i>Escherichia coli</i>	1.25–2.5	0.3–3.75
<i>E. coli</i> R†	1.5–5.0	>1,000
<i>Hemophilus influenzae</i>	1.25–2.5	1.56–5.0
<i>H. pertussis</i>	2.5	1.25–3.0
<i>Klebsiella pneumoniae</i>	0.312–0.625	0.625–8.0
<i>K. pneumoniae</i> R	0.312	>1,000
<i>Malleomyces mallei</i>	>10.0	10–>10.0
<i>Mycobacterium avium</i>	0.1–0.3	10
<i>M. phlei</i>	0.05–0.078	0.12
<i>M. tuberculosis</i>	<0.5	1.0–5.0
<i>M. tuberculosis</i> R	<0.5	>100
<i>Neisseria intracellulæ</i>	1.25–2.5	5.0
<i>Pasteurella pestis</i>	0.625	0.75–1.5
<i>P. tularensis</i>	0.156	0.15–0.3
<i>Phytomonas pruni</i>	0.1	0.25
<i>Proteus vulgaris</i>	1.25–2.5	0.4–3.0
<i>Pseudomonas aeruginosa</i>	12.5–25.0	2.5–25.0
<i>Salmonella typhosa</i>	0.1–0.625	1.0–37.5
<i>S. schottmülleri</i>	0.4–0.7	2.0
<i>Sarcina lutea</i>	2.5	0.25
<i>Serratia marcescens</i>	1.25	1.0
<i>Shigella paradysenteriae</i>	0.25–0.5	0.25–3.75
<i>Staphylococcus aureus</i>	0.156–0.625	0.5–>16.0
<i>Streptococcus faecalis</i>	5.0	50.0
<i>Vibrio comma</i>	2.5	6.0–37.5
Various fungi	>10.0	>10.0

* Based on results obtained by F. Heilman at Mayo clinic; O. Graessle at Merck Institute; and in our own laboratories.

† Results reported by Waksman, S. A., and Schatz, A., Am. Pharm. Assoc., 1945, 34, 273.

‡ Streptomycin-resistant.

antibiotics, emphasized the fact that we were dealing with a new type of antibiotic substance. It was, therefore, designated as *Neomycin*.

Neomycin was found to be less favorable to the development of resistant strains of bacteria on contact with it than is usually found to be the case with streptomycin. A 20-hour-old agar culture of *E. coli* was suspended in water and plated out in nutrient agar containing varying amounts of neomycin. Of 22 billion cells added to each plate, only very few colonies developed. When pieces of agar were removed from the plates and added to sterile media, only the 5 u./ml. plate gave any bacterial growth; the 10 u./ml. and 25 u./ml. agar

plates gave no growth, thus pointing to the high bactericidal properties of neomycin. Figure 1 and Table VI illustrate the difference in the development of bacterial resistance to neomycin and streptomycin.

Plates containing varying amounts of neomycin were streaked with streptomycin-sensitive, -resistant and -dependent strains of *E. coli* (4); the

first two strains were found to be sensitive alike to neomycin, and the last made no growth. This established further the marked difference in antibacterial behavior of neomycin and streptomycin. A comparison of the sensitivity of many strains of the same organism to neomycin revealed considerable variations. Most strains of *E. coli*, for example, were sensitive to 2.5 u./ml.; one

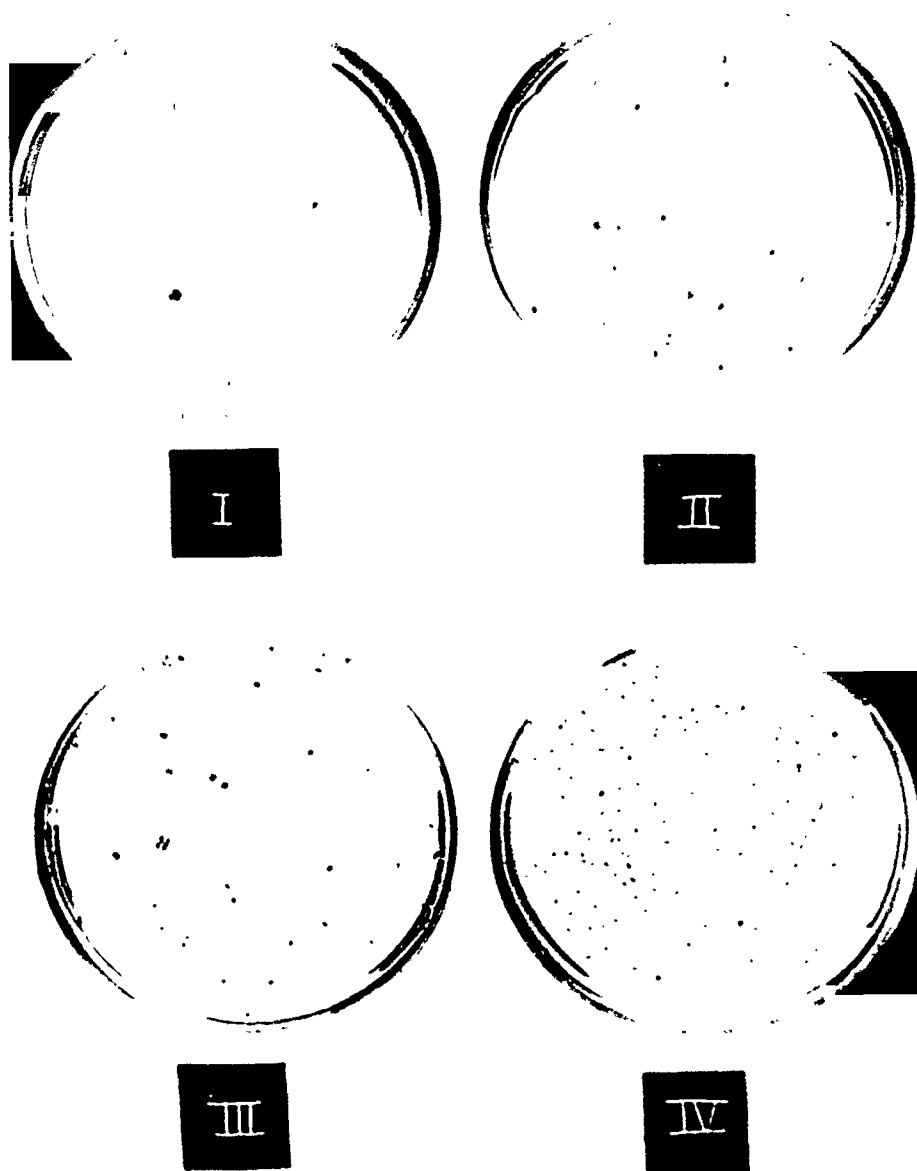


FIG. 1. RELATIVE SURVIVAL OF *E. coli* CELLS IN AGAR MEDIA CONTAINING NEOMYCIN AND STREPTOMYCIN

Number of cells added per plate I and II 6.7×10^6 . Number of cells added to plates III and IV 6.7×10^7 . Plates I and III contain 4 u. neomycin/ml.; plates II and IV contain 4 u. streptomycin/ml.

TABLE VI

Survival of E. coli in plates containing varying concentrations of neomycin and streptomycin

Incubation of plates	Neomycin (u./ml.)				Streptomycin (μg./ml.)			
	2	4	6	8	2	4	6	8
	Colonies developing on plate from 1 ml. of bacterial suspension*							
hrs.								
24	700	8	0	0	21,000	270	20	0
48	1,500	65	1	<1	4,600,000	560	70	26
72	1,700	108	2	<1	6,700,000	640	130	70
120	1,700	108	2	<1	7,400,000	640	130	70

* 1 ml. of 24-hour-old bacterial suspension contained 246 million cells.

strain, however (ATCC 6880), was resistant to 5 u./ml.

When broth or agar cultures of various bacteria containing sufficient neomycin to inhibit growth were incubated for longer periods, no further development of the bacteria occurred, thus pointing to the stability of this antibiotic, as contrasted to aureomycin, for example. Similar results were obtained with saprophytic mycobacteria and the pathogenic *M. tuberculosis*.

Identity of neomycin-producing organism 3535

The neomycin-producing culture was found to be quite distinct in its cultural and other properties from the other known antibiotic-producing species of *Streptomyces*. Its growth on synthetic and organic media was yellowish to brownish, but no soluble pigment was formed, thus placing it among the non-chromogenic forms. Aerial mycelium was readily produced on synthetic media; it was pigmented at first white, turning to rose-pink or flesh-pink or seashell pink (light-russet-vinaceous, according to Ridgway, XXXIX b-9"). On certain media, the mycelium tended to be patchy, gradually covering the surface of the vegetative growth. The sporulating hyphae were usually straight, either forming no spirals or only an occasional loose spiral. Among the type cultures already described in the literature, the above description was found to fit best with the organisms described by Waksman and Curtis as *Actinomyces* (*Streptomyces*) *fradiae* (5).

No. 3535 produced excellent growth in shaken cultures; it frequently tended to undergo lysis, especially in poorly buffered media. It has not

been established as yet whether the lysis of the culture is due to infection with phage or to the action of an autolytic enzyme. In contrast to streptomycin, once neomycin was produced, however, it remained in the medium and was not readily destroyed.

Physical and chemical properties of neomycin

A detailed survey of the physical and chemical properties of neomycin will be presented elsewhere. It is sufficient to summarize some of these properties here.

Neomycin is highly resistant to the action of micro-organisms. It is heat-stable and is resistant to the action of acid (pH 2.0) at the temperature of boiling water.

Neomycin is favored in its antibacterial activity by an alkaline reaction of the medium. The best results were obtained with a pH of 7.0–8.0 of the agar and a pH of 7.0 of the buffer. The presence of glucose in the test medium reduces the potency of the antibiotic by favoring either acid production or growth of test organism.

Cysteine has apparently no marked effect upon the activity of neomycin. When 1 to 10 mg. of cysteine are added to 50 u. of neomycin in a phosphate buffer at pH 7.0 and solutions allowed to stand at room temperature for three to 20 hours, only a slight loss in activity occurs.

SUMMARY

The formation of a new antibiotic, designated as neomycin, by a culture of *Streptomyces* (No. 3535) closely related to *S. fradiae* is reported.

Neomycin is produced, under shaken or submerged conditions, in media similar to those used for the production of streptomycin by *S. griseus*. The culture tends at first to form acid and undergo lysis. This can be prevented by addition of CaCO_3 to sugar-rich media, or by reducing the sugar content of the medium, or by increasing the peptone content. Addition of a small amount of zinc has a favorable effect.

Neomycin belongs to the basic group of antibiotics, which includes a number of substances already described in the literature.

Neomycin is heat-stable. It is also stable to the action of micro-organisms. It is favored in its activity by an alkaline reaction of the medium.

It is not favored by the presence of glucose in the medium.

Neomycin is active against a large variety of bacteria, including Gram-positive and Gram-negative, as well as acid-fast, forms. It is active alike against streptomycin-sensitive and streptomycin-resistant strains of bacteria, including those of *M. tuberculosis* var. *hominis*. It is not active against fungi.

Neomycin is not only bacteriostatic but also strongly bactericidal. It does not readily allow development of resistant strains of bacteria among the sensitive forms.

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DIFFUSION PLATE ASSAY FOR CHLORAMPHENICOL AND AUREOMYCIN¹

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In the development of bioassay methods for antibiotics a certain standard pattern is usually followed. First, a wide variety of microorganisms are tested for their sensitivity to the new drug and then sensitive strains are selected and tested for their suitability for use in a plate assay, turbidimetric assay and serial dilution assay. Since the Food and Drug Administration tests a number of antibiotics, every effort is made to adapt test procedures for new antibiotics to those already in use. Thus, in the two plate assay methods to be described, it has been found possible to utilize the same test organism; the same medium as that used in the seed layer for the penicillin plate assay; and the general procedure for determining the potency of an unknown which is the same as that described for penicillin in the Federal Register of June 3, 1948.

The percentage error of the assay may be determined by the double dose technic described by Knudsen and Randall (1). A 1:4 ratio is used for chloramphenicol (20 μ g. and 80 μ g.), and a 1:10 ratio for aureomycin (3 μ g. and 30 μ g.). The chart and nomograph for the 1:10 ratio are not given in the above publication but may be obtained on request from the Food and Drug Administration. The percentage error of the chloramphenicol plate assay has averaged 2.23% on about 21 determinations with a range of from 0.7% to 4.58%. The error of the aureomycin assay is somewhat higher. In 24 determinations the percentage error ranged from 2.5% to 14.8% with an average of 7.4%. In our experience the cup-plate method for chloramphenicol is the most accurate and reproducible one yet encountered. A composite curve was prepared from ten daily chloramphenicol curves and the potency of samples previously run recalculated. Since no significant differences in potency were noted, a daily curve is

no longer necessary; however, the composite curve is checked against a new standard curve once every week. A description of the methods follows.

Culture media. Use ingredients that conform to the standards prescribed by the U.S.P. or N.F. Make nutrient agar for the seed and base layer and for carrying the test organism as follows:

Peptone	6.0 gm.
Pancreatic digest of casein	4.0 gm.
Yeast extract	3.0 gm.
Beef extract	1.5 gm.
Glucose	1.0 gm.
Agar	15.0 gm.
Distilled water, q.s.	1000.0 ml.
pH 6.5 to 6.6 after sterilization	

Make nutrient broth, for preparing a suspension of the test organism, as follows:

Peptone	5.0 gm.
Yeast extract	1.5 gm.
Beef extract	1.5 gm.
Sodium chloride	3.5 gm.
Glucose	1.0 gm.
Dipotassium phosphate	3.68 gm.
Potassium dihydrogen phosphate ...	1.32 gm.
Distilled water, q.s.	1000.0 ml.
pH 7.0 after sterilization	

In lieu of preparing the media from the individual ingredients specified above, they may be made from a dehydrated mixture which, when reconstituted with distilled water, has the same composition as such media.

Working standard

A. Chloramphenicol. Weigh out carefully appropriate amounts of the standard which is crystalline synthetic chloramphenicol and dilute in 1% phosphate buffer pH 6 to give a solution containing 100 μ g. per ml. Keep this stock solution at a temperature of 15° C. or less, and use for only one week. From this stock solution make appropriate working dilutions in the above buffer. To facilitate solutions, the standard may be first dissolved in a small amount of ethyl alcohol.

B. Aureomycin. Proceed as above except dilute the aureomycin standard which is crystalline aureomycin to 1000 μ g. per ml. in 1% potassium acid phthalate-tartaric acid buffer pH 3.0 and use for only three days.

Preparation of sample.

A. Chloramphenicol. Dissolve the sample to be tested in a small amount of ethyl alcohol and then further dilute

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in 1% phosphate buffer pH 6 to make an appropriate stock solution.

B. Aureomycin. Dissolve the sample to be tested in sterile distilled water to make an appropriate stock solution.

Preparation of plates

I. Base layer. Add 21 ml. of agar to each Petri dish (20 × 100 mm.). Distribute the agar evenly in the plates and allow it to harden. Use the plates the same day they are prepared.

II. Seed layer. The test organism is *Sarcina lutea* (P.C.I. 1001). Maintain the test organism on slants of nutrient agar prepared as described above, and transfer to a fresh agar slant once a week. Prepare a suspension of the test organisms as follows: Streak an agar slant heavily with the test organism. Incubate 24 hours at 26° C. Wash the growth off in about 3 ml. of nutrient broth. Use the suspension so obtained to inoculate the surface of a Roux bottle containing 300 ml. of the above agar. Spread the suspension over the entire surface with the aid of sterile glass beads. Incubate 24 hours at 26° C.

A. Chloramphenicol. Wash the resulting growth from the agar surface with about 20 ml. of nutrient broth. If an aliquot of this bulk suspension, when diluted with nutrient broth 1:10, gives a 10% light transmission, the bulk suspension is satisfactory for use in the test. It may be necessary to dilute the bulk suspension so that a 1:10 dilution of an aliquot gives a light transmission of 10%. A photoelectric colorimeter equipped with a filter having a wave length of 6500 Angstrom units is used in the standardization. The bulk suspension may be used in the test for one week or longer. Add 1.5 ml. of the adjusted bulk suspension to 100 ml. of agar which has been melted and cooled to 48° C. Use 4 ml. per plate of this inoculum for the seed layer.

B. Aureomycin. Wash the growth from the agar surface with 50 ml. of nutrient broth. If an aliquot of this bulk suspension when diluted 1:50 in saline gives 75% light transmission (filter same as above), the bulk suspension is satisfactory for use in the test. As above, it may be necessary to dilute the bulk suspension so that an aliquot gives 75% light transmission. The bulk suspension may be used in the test for at least one week. Add 0.3 ml. of the adjusted bulk suspension to 100 ml. of agar which has been melted and cooled at 48° C. Use 4 ml. per plate of this inoculum for the seed layer.

Assay

A. Chloramphenicol. The potency of the sample is determined by the standard curve technic, using a single dose of standard and unknown.

Dilute the sample to be tested to an estimated 50 µg. per ml. in 1% phosphate buffer pH 6. Place six cylinders on the inoculated agar surface so that they are at approximately 90° intervals on a 2.8 cm. radius. Use three plates for each sample.

Fill three cylinders on each plate with the 50 µg. per ml. standard and three cylinders with the 50 µg. per ml. (estimated) sample, alternating standard and sample. At the same time prepare a standard curve, using concentrations of the standard of 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0, and 70.0 µg. per ml. A total of 24 plates is used in the preparation of the standard curve, three plates for each solution except the 50 µg. per ml. solution. The latter concentration is used as the reference point and is included on each plate. On each of three plates fill three cylinders with the 50 µg. per ml. standard and the other three cylinders with the concentration of the standard under test. Thus, there will be seventy-two 50-µg. determinations and nine determinations for each of the other points on the curve. Incubate the plates for 16 to 18 hours at 37° C. and measure the diameter of each circle of inhibition. Average the readings of the 50 µg. per ml. concentration and the readings of the point tested for each set of three plates, and average also all 72 readings of the 50 µg. per ml. concentration. The average of the 72 readings of the 50 µg. per ml. concentration is the correction point for the curve. Correct the average value obtained for each point to the figure it would be if the 50 µg. per ml. reading for that set of three plates were the same as the correction point. Thus, if in correcting the 40 µg. per ml. concentration the average of the 72 readings of the 50 µg. per ml. concentration is 18.0 mm., and the average of the 50 µg. per ml. concentration of this set of three plates is 17.8 mm., the correction is .2 mm. If the average reading of the 40 µg. per ml. concentration of these same three plates is 17.0 mm., the corrected value is then 17.2 mm.

Plot these corrected values including the average of the 50 µg. per ml. concentrations on two-cycle semi-log paper, using the concentration in µg. per ml. as the ordinate (the logarithmic scale) and the diameter of the zone of inhibition as the abscissa. Draw the standard curve through these points.

To estimate the potency of the sample, average the zone readings of the standard and the zone readings of the sample on the three plates used. If the sample gives a larger zone size than the average of the standard, add the difference between them to the 50 µg. per ml. unit zone on the standard curve.

ard curve. If the average value is lower than the standard value, subtract the difference between them from the 50 $\mu\text{g.}$ per ml. unit value on the curve. From the curves read the potencies corresponding to these corrected values of zone sizes.

B. Aureomycin. The assay for aureomycin follows very closely that described for chloramphenicol. The following exceptions are noted.

1. Dilute the stock solution of the sample to be tested to 10.0 $\mu\text{g.}$ per ml. (estimated) in 1% potassium acid phthalate-tartaric acid buffer (pH 3.0).

2. Prepare a standard curve, using concentrations of the standard of 4.0, 6.0, 8.0, 10.0, 13.0, 17.0, 22.0, and 29.0 $\mu\text{g.}$ per ml. in 1% potassium acid phthalate-tartaric acid buffer pH 3.0. The

10 $\mu\text{g.}$ per ml. concentration is the reference point for the aureomycin curve, and the correction point is obtained by averaging the readings of all of the 10 $\mu\text{g.}$ per ml. concentrations.

SUMMARY

1. A cup-plate assay for chloramphenicol and aureomycin is described.

2. The organism, medium and the general method for estimating the potency are the same for both antibiotics.

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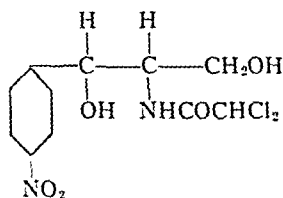
CHLORAMPHENICOL (CHLOROMYCETIN), AN ANTIBIOTIC. PHARMACOLOGICAL AND PATHOLOGICAL STUDIES IN ANIMALS^{1, 2, 3}

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Chloramphenicol is an antibiotic which initially was isolated from culture filtrates of the fungus *Streptomyces venezuelae* (1-3) by Bartz (4) and subsequently was synthesized chemically (5-7).

Chloramphenicol (Chloromycetin) is a pure chemical substance, *D*(-)-*threo*-1-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol, and has the following chemical structure



It is a neutral white crystalline substance with a bitter taste and is stable in neutral and acid solutions. The drug is moderately soluble in water (0.25 per cent at room temperature, 0.65 per cent at 70° C and up to 15 per cent in propylene glycol, 5 per cent in 50 per cent acetamide), and is insoluble in vegetable oils. Solubility in urine or serum is about the same as in water. Solutions are stable on heating.

Material for this study consisted of crystalline chloramphenicol obtained from culture filtrates, and the synthetic product.

Chloramphenicol was administered intravenously in aqueous propylene glycol or acetamide solutions to small animals at a rate of 0.1 cc. per ten seconds; dogs received 1.0 to 3.0 cc./min. Acute toxicity results are based on a seven- to 14-day observation period and chronic tolerance studies were carried out for two to four weeks in small animals and for periods of three and five weeks and over four months in dogs. The Dragstedt *et al.* (8) method served to interpolate results.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² Chloramphenicol is a generic name coined from the chemical structure of the compound.

³ Chloromycetin is a designated trade mark under which it was originally studied in the laboratory.

Acute Toxicity

Intravenous administration of a 0.5 per cent aqueous solution of chloramphenicol to 20-gram white mice caused slight transient ataxia with 125 to 150 mg./kg. doses. At lethal or near-lethal doses of 175 to 300 mg./kg., the mice became incoordinated, some were flaccidly prostrate and dyspneic, and death occurred within a few minutes from respiratory failure. The surviving animals appeared normal within a few minutes to one-half hour. Intraperitoneal and oral administration of toxic doses produced similar reactions. Perorally in single doses mice showed a slight dyspnea with 1.5 grams/kg. and dogs were free of reactions at 150 to 200 mg./kg. once or twice daily. Administration of a two-fold increase in the above amounts to dogs produced sporadic vomiting, acute diarrhea, irritability, occasional spasticity and clonic convulsive seizures. The comparative toxicities of the fermentation and synthetic products were nearly identical (Table I).

Intravenous administration of chloramphenicol to adult dogs in 60 to 87 per cent propylene glycol solution at a rate of 3 cc./min. caused transient rise of body temperature of 0.6 to 1.7° F. corresponding to temperature changes from equivalent amounts of propylene glycol alone. The pulse rate had a tendency to become slightly depressed, while the respiration rate increased. A transient hematuria appeared with 150 mg./kg. doses without progressive histopathologic changes in the kidneys. The hemogram was not altered in a seven-day observation period.

When chloramphenicol as a 10 per cent solution in propylene glycol was administered repeatedly at 30-minute intervals, 1 cc. min., intravenously to dogs under pentobarbital anesthesia and the carotid blood pressure and respiratory excursions were recorded by conventional methods,* no alteration in blood pressure or amplitude of excursions or in respiratory rate occurred at 125 to 250 mg./kg.

*By Graham Green.

TABLE I

Acute parenteral and oral toxicity of chloramphenicol prepared synthetically or by fermentation

Animal	Route	Material and diluent	Number animals	M.T.D.* LD ₅₀	LD ₅₀
				mg./kg.	mg./kg.
Albino Mice	I.V.	Fermentation, 25-28% propylene glycol	455	50	109.5
	I.V.	Fermentation, water	964	125	195.4
	I.V.	Synthetic, water	375	125	202.6
	I.P.	Fermentation, acacia-water suspension	280	750	1320.0
	I.P.	Fermentation, water	170	625	1320.0
	Oral	Fermentation, acacia-water suspension	270	1500	2640.0
Albino Rats	I.V.	Fermentation, 60% propylene glycol	438	100	175.5
	I.V.	Synthetic, 60% propylene glycol	239	100	170.5
	I.V.	Fermentation, 50% acetamide	518	200	279.4
	I.V.	Synthetic, 50% acetamide	140	225	278.0
Dogs	I.V.	Fermentation, 75-85% propylene glycol	7	150**	—
	I.M.	Fermentation, peanut oil suspension	3	>101	—
	I.M.	Fermentation, 62% propylene glycol	8	>46.5	—
	Oral	By capsule, powder	7	>300	—
Rabbits	I.V.	Fermentation, 100% propylene glycol	25	75	117.0

* Maximal tolerated dose (M.T.D.)—Survival of 100 per cent of animals.

** Renal hemorrhage was frequent.

doses of chloramphenicol. An 11 and 71 per cent decrease in blood pressure occurred with 50 and 100 mg./kg. doses respectively and this was accompanied by slight increase in respiration rate and decrease in amplitude. Single doses to individual animals caused no blood pressure changes with 12.5 and 25 mg./kg., a fall of about 7 to 10 per cent was produced with 50 and 100 mg./kg. Increasing the injection rate had a tendency to cause a "speed-shock" and a 150 mg./kg. dose at 4 cc./min. produced a 97 per cent fall in blood pressure and death from respiratory failure. Analogous volumes of propylene glycol alone caused 5 to 10 per cent fall in blood pressure on repeated injections in the same animal and none when given in single doses to different animals (Figures 1 and 2).

Chronic Toxicity

In chronic tolerance studies different dose levels of chloramphenicol were administered to different groups of mice, guinea pigs, rats, rabbits, and dogs. All animals were observed daily for food consumption, reactions and weekly body weight changes. Blood counts were made on guinea pigs, rats and dogs. The blood non-protein nitrogen, bromsulphalein liver and phenolsulphathiazole kidney functions, urine analysis for albumin and sugar, and methemoglobin values were determined in dogs. The blood and urinary concentration of chloramphenicol was determined on mice, guinea pigs and dogs.

Urine volume excretion was determined in mice and dogs. The maximal tolerated doses (M.T.D.) which caused no reactions and produced only slight or no depression of normal body weight gain in animals and the 50 per cent lethal doses (LD₅₀) are shown in Table II.

Subcutaneous injections of 50 to 100 mg./kg. twice daily in 10-20 per cent propylene glycol solution to 20-gram mice for 14 days showed a slight to 10 per cent weight gain depression. With 200 and 400 mg./kg. doses, the mice refused food and lost weight, and death of 20 and 100 per cent of animals occurred on respective doses. The sites of repeated injections became indurated and ulcerated. Death was due to anorexia and cachexia, complicated by ulcerations penetrating the abdominal wall.

When chloramphenicol was given to 20-gram mice in the diet, normal growth of animals was maintained on 0.125 and 0.25 per cent concentrations (203 and 385 mg./kg. per day) but with 0.5, 1.0 and 2.0 per cent concentrations (respectively 679, 1290 and 2060 mg./kg. daily) for a two-week period, mice lost from 0.85 to 4.0 grams in weight from anorexia without showing other reactions.

Oral administration to mice by cannula in acacia-water suspension of about 215 mg./kg. twice daily for four weeks caused no anorexia and allowed normal growth rate. A 311 mg./kg. dosage

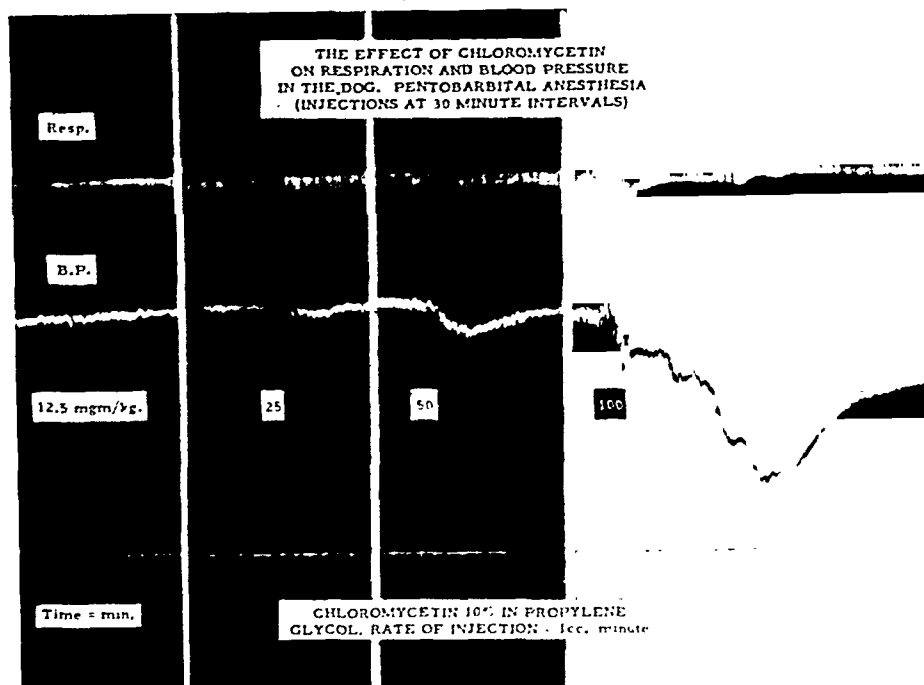


FIG. 1. INTRAVENOUS INJECTIONS OF CHLORAMPHENICOL IN ANESTHETIZED DOGS

Repeated injections caused no blood pressure changes with 12.5 and 25 mg./kg. A slight depression occurred with 50 mg./kg. and a rapid fall with 100 mg./kg. accompanied by decrease in amplitude and increase of respiratory rate. Increasing speed of injection to 4 cc./min. a 150 mg./kg. dose produced 97 per cent fall in blood pressure and death from respiratory failure.

twice daily produced death of 30 per cent of the animals. It was evident that daily oral intakes in mice of 385 to about 425 mg./kg. in divided doses constituted about the maximal tolerated amounts of chloramphenicol, whether administered in the food or by cannula.

Guinea pigs had considerable aversion to the diet (rabbit chow pellets) (9) containing chlo-

ramphenicol. This was overcome in part by starting animals first on 0.125 per cent (90[±] mg./kg. daily) and increasing the concentration for the second week to 0.25 per cent (153[±] mg./kg.) and to 0.5 per cent (256[±] mg./kg.) in the third week. On this schedule, guinea pigs maintained or increased their weight. When placed initially on a

[±] Actual amount ingested per day.

TABLE II
Chronic toxicity of chloramphenicol

Animal	Mode and duration of administration	M.T.D.	L.D. ₅₀
Albino Mice	In diet ration, daily ingestion for 2 weeks	385	> 2000
	By cannula, in two divided doses daily for 4 weeks	425	> 622
	Subcutaneously in propylene glycol daily for 2 weeks	100	275
	Intraperitoneally in water once daily for 2 weeks	250	> 1000
Guinea Pigs	In diet ration, daily ingestion for 2 to 4 weeks	250 (0.5%)	518 (1.0%)
Dogs	Orally by capsule for 3 and 5 weeks and over 4 months in two divided doses daily	> 200	
	I.M. in propylene glycol for 24 days in two divided doses daily	> 74.93	
	Orally by capsule for 7 days in single daily doses	> 300	
Rabbits	I.M. in propylene glycol for 8 days in two divided doses daily	> 100	

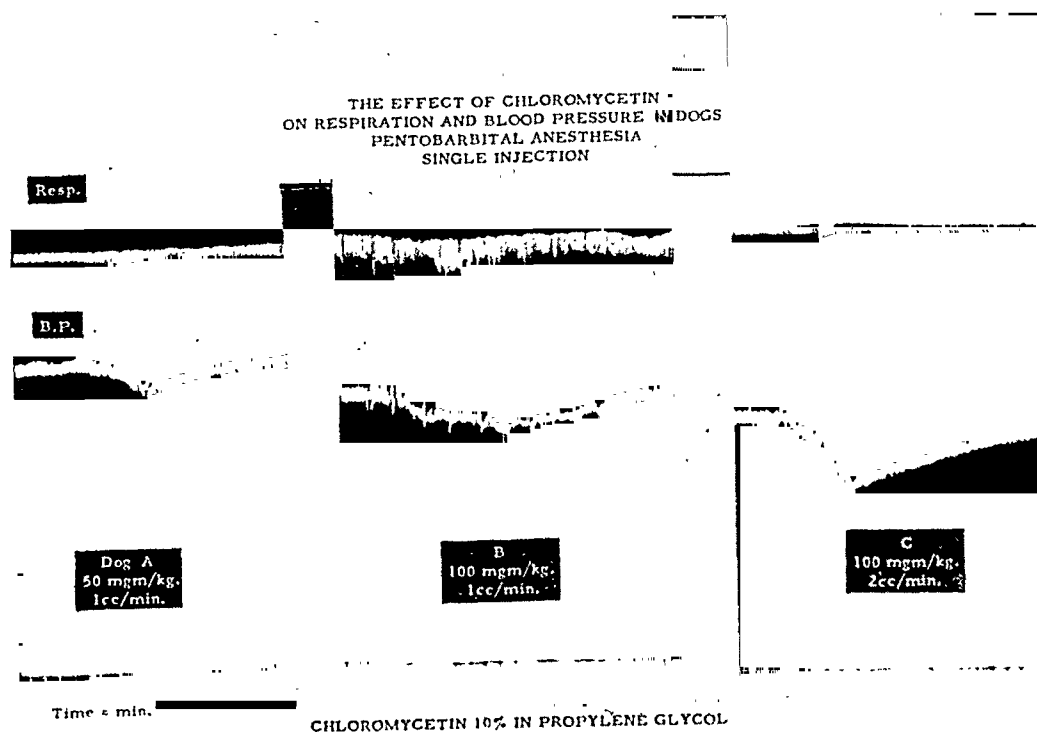


FIG. 2. INTRAVENOUS INJECTION OF CHLORAMPHENICOL IN ANESTHETIZED DOG(S)

Single injections of 50 and 100 mg./kg. at a rate of 1 cc./min. caused a slight blood pressure fall (7 to 10 per cent); doubling the injection rate caused the blood pressure to fall about 31 per cent.

0.5 per cent diet concentration, the animals consumed about one-third of the normal food intake for the first week and about three-quarters of normal subsequently. On a 1.0 per cent concentration in the diet, they consumed about one-fourth of normal food intake, lost on an average of 117 grams in weight in two weeks and six out of eight died from cachexia. The two surviving guinea pigs were cachectic, showed a slight hemoglobin concentration (120 per cent versus controls of 104 per cent), with rise of neutrophils to 77 per cent (controls 44 per cent), and the total white cell count remained about unchanged.

Intramuscular Tolerance in Dogs

A group of three young, recently acquired, street dogs weighing 11 to 14 kg. each and showing initial clinical symptoms of "distemper" (a slight body temperature rise, conjunctivitis and serous rhinitis) were each injected intramuscularly twice daily with 0.5 gram of the fermentation process chloramphenicol partly dissolved in 2 cc. of 62 per cent propylene glycol for 38 doses in 24 days. In the first seven to ten days of treatment the temperature of the dogs rose to 103.2 to 106° F. and

receded to normal at ten, 19, and 21 days respectively under treatment. During the fever period the dogs developed anorexia and considerable anemia with 2.5, 3.4 and 4.2 million erythrocytes per cu. mm. respectively. The red cell counts rose to 4.0, 3.4 and 5.5 million as the fever declined and the appetite of the dogs improved. Injections were followed by considerable to severe local tissue induration, extensive edema, hemorrhage and necrosis. Tentatively it was assumed that the anemia was associated with "distemper" and probably the severe local tissue changes were a contributory factor. Autopsy and microscopic tissue examination showed no significant pathologic changes in the liver, spleen, kidneys and other visceral organs, while the lungs showed residual inflammatory foci.

Oral Tolerance in Dogs

Initially 800 mg. or 86 mg./kg. of the fermentation process crystalline chloramphenicol was given to a dog, a single dose orally in a gelatin capsule. The animal remained free of systemic or gastrointestinal reactions. Two dogs were given 300 mg. kg. daily as a single dose for four days. Ani-

mals showed anorexia, lost weight and vomited occasionally. Rise of body temperature of 0.8 to 1.4° F. occurred on the first day; the temperature was normal subsequently. Two other dogs received 200 mg./kg. twice daily for 32 doses in 21 days. These animals developed severe anorexia, adipsia and lost 2.5 to 4.5 kg. in body weight. At the end of the third week the urine showed casts and a large amount of albumin. The body temperature remained normal. Another dog, fed 0.5 gram (73 mg./kg.) twice daily for 38 doses in 24 days, remained asymptomatic. This animal was studied for hematologic, bromsulfalein liver function, blood sugar and total non-protein nitrogen changes. The various test results showed normal values, and the urine remained free of albumin and sugar. Three additional dogs were given 75 mg./kg. twice daily of the fermentation product for 66 doses in 39 days and seven dogs were given synthetic chloramphenicol, 50, 75 and 100 mg./kg. twice daily respectively for 194 doses in 133 days. The dogs of the former group were fed chloramphenicol six days, and the latter five days each week. The daily dosage for each dog was adjusted at weekly intervals to the gain or loss in body weight. The animals of both groups remained in good physical condition; the first group gained 0.55 to 0.85 kg., the second group, 0.45 to 2.75 kg., while two animals remained about stationary (-0.25 to +0.3 kg.). Gastro-intestinal

reactions occurred rarely; one dog vomited once after the 30th dose (50 mg./kg.), the second animal vomited once after each of the 153rd and 166th doses, and a third animal after the 173rd dose (100 mg./kg.). No diarrhea was encountered. The animals showed no disturbances of vision, hearing or somatic reflexes. The urine remained free of sugar and albumin, but occasionally following catheterization injury, blood appeared in urine. The body temperature remained normal except in one animal following catheterization injury and ensuing cystitis. Blood pressures were determined three times on each dog of the second group by an indirect tail plethysmograph method and the readings fluctuated in a normal range of 135 to 165 mm. of mercury, while six untreated dogs showed blood pressure range of 135 to 172 mm. Examination of tissues at autopsy revealed no gross pathologic changes, except focal inflammatory hemorrhagic areas of the urinary bladder of an animal with chronic ulcerative cystitis. Volume of daily urine excretion was in the range of normal dogs.

The hematology in these dogs remained relatively unchanged, although roughly about 300-350 cc. of blood were withdrawn from each dog for chloramphenicol determinations, N.P.N. and blood count studies (Figure 3). In one of these dogs the red cell counts decreased to 4.9 million per cu. mm. and hemoglobin values to 85 per cent. Autopsy revealed ulcerative cystitis and atrophic

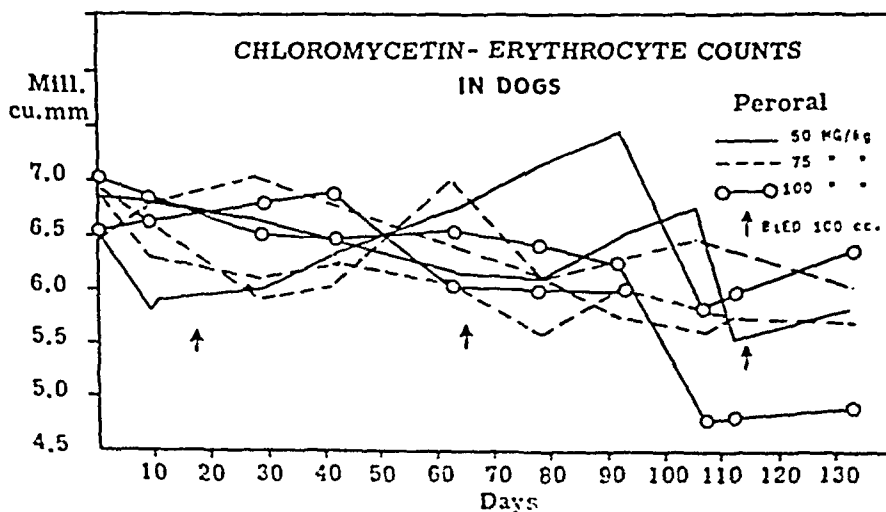


FIG. 3. RED CELL COUNTS IN DOGS WHICH RECEIVED CHLOROMYCETIN FOR 194 DOSES IN 134 DAYS AND SUBJECTED TO FREQUENT BLOOD COUNTS. IN CLINICAL STUDIES REMAINED ESSENTIALLY AT NORMAL LEVELS.

stomach mucosa. The leucocyte counts of treated animals remained in normal range and varied slightly from the pre-treatment values of 7,400 to 15,900, average of 12,400, to the last counts of 10,200 to 14,100 per cu. mm., average of 12,900. The animal with chronic cystitis and atrophic gastritis had an initial count of 18,200 leucocytes per cu. mm. and it remained at this level until the last three weeks when the leucocytes rose to 33,000 and the temperature to 103.3° F. The total blood non-protein nitrogen in the latter animal remained in a normal range (32-46 mg. per cent). The differential counts in the above dogs ranged from 71 to 90 per cent neutrophils prior to chloramphenicol administration and during the treatment period 80 to 90 per cent neutrophils, 10 to 17 per cent lymphocytes and scattered eosinophils and mononuclear cells. No tendency to agranulocytosis or abnormal cell morphology was observed.

The total blood non-protein nitrogen was determined in the morning before dosing, five to nine times on each dog. In this group of dogs the normal values ranged between 19 and 50 mg. per cent. Under chloramphenicol treatment non-protein nitrogen values showed sporadic fluctuation (Table III) which was not parallel to size of dosage or duration of treatment. Moreover, the fluctuation in values was not regularly sustained or cumulative, but paralleled the amount of meat

in the diet ration ingested previous to sampling.

Bromsulfalein liver function tests indicated dye retentions of less than 5 per cent in 30 minutes. Phenolsulfonphthalein excretion amounted to about 58 per cent at one hour as compared with 52 per cent in the pre-treatment period. Daily water intake was reduced by about 50 per cent of normal animals and the urine volume in treated dogs fluctuated from 21 to 32 cc./kg. in 24 hours as compared with 20 to 60 cc./kg. in the normal control period. Anuria was not in evidence, and the renal clearance of creatinine remained normal.

Pathology

Pathologic tissue changes in non-toxic dose range of chloramphenicol were essentially absent. In the toxic range the pathology was related to a "shock-like" effect of chloramphenicol, resulting in prostration, severe respiratory depression or failure, fall of blood pressure, thready pulse, and anoxia. The visceral organs were congested with scattered petechial hemorrhages; the kidneys were moderately swollen and renal hemorrhage occurred on intravenous administration, probably due to propylene glycol (10). The animals either died from respiratory failure within a few minutes after intravenous administration or within two hours on oral administration. The tissues of surviving animals, at the end of the seven-day observation period, on microscopic examination, were essentially free of acute degenerative changes. Tissues of dogs on daily oral intake of 100 to 200 mg./kg. for over four months were free of acute or cumulative chronic degenerative changes except some hydropic changes in capillaries of the glomerulae. Since renal hemorrhage occurred on intravenous administration of maximal tolerated doses of propylene glycol solutions of chloramphenicol, the differential histopathologic changes are under further investigation and will be reported on at a later date.

Blood Serum and Urinary Concentration and Excretion

A four-hour turbidimetric method (11), employing *Shigella sonnei* as a test organism, was used to determine chloramphenicol concentration in blood serum, urine, bile and spinal fluid. One µg./cc. is the threshold of the test sensitivity. In-

TABLE III

Total blood non-protein nitrogen in chloramphenicol-fed dogs

Dogs	1	2	3	4	5	6	7	8	9	10	11
Chloramphenicol in milligrams per kilogram twice daily											
	75	75	75	75	50	50	75	75	75	100	100
Total blood non protein nitrogen, milligrams per cent											
0	28	34	19	30	31	34	42	29	50	24	21
3	36	42		44	35						
10	51	27	26	21		39	58	35	60	27	40
17	54			32							
32		23	36	47	43	45	39	31	48	44	43
38		53	43	46					53		43
63					38	36	35	45		49	41
78					50	43	30	59	34	44	39
92					44	49	31	40	34	40	44
105					36		30	45	28	50	
112					41	46	48		26	46	42
133					39	35	48	47	53	42	32
Average	48	36	35	36	41	42	46	43	42	42	40

travenous administration produced initial blood serum concentration of 17 to 45 $\mu\text{g./cc.}$ at 15 minutes, roughly proportional to 19 and 50 mg./kg. dosage. The serum levels rapidly decreased to below 1.0 $\mu\text{g./cc.}$ at four and six hours respectively (Figure 4). Intramuscular administration in peanut oil suspension of 90 to 101 mg./kg. produced serum levels of 1.5 to 7.5 $\mu\text{g./cc.}$ for a seven-hour period and less than 1 $\mu\text{g./cc.}$ at the 16th hour. When chloramphenicol in propylene glycol was given twice daily intramuscularly in doses of 37 to 46.5 mg./kg., the serum levels varied from 0 to 4 $\mu\text{g./cc.}$ at two hours after the first dose, 3 to 6 $\mu\text{g./cc.}$ at two hours after the second daily dose, and 0.0 to 1.0 $\mu\text{g./cc.}$ on the following morning. Oral administration in dogs of single doses of 86 and 150 mg./kg. provided serum levels from 8 to 39 $\mu\text{g./cc.}$ at two hours, 10.6 to 20 $\mu\text{g./cc.}$ at eight hours and less than 1 $\mu\text{g./cc.}$ at 12 hours. Dogs treated perorally twice daily for over four months were sampled four times at different 24-hour intervals and the average serum levels for an eight-hour period ranged from 6.2 to 9.8 $\mu\text{g./cc.}$ for the 50 mg./kg. dose, 19.1 to 25.8 $\mu\text{g./cc.}$ for the 75 mg./kg. dose and 26.6 to 36.4 $\mu\text{g./cc.}$ for the 100 mg./kg. dosage. The blood

serum contained no chloramphenicol 18 hours after the second daily oral dose. Bile contained 10 to 30 $\mu\text{g./cc.}$ at two hours after dosing and cerebrospinal fluid 0 to 2.5 $\mu\text{g./cc.}$ after the first and 3 to 4 $\mu\text{g./cc.}$ after the second 75 mg./kg. daily dosing. A dosage of 200 mg./kg. twice daily showed serum levels of 80, 120, 20 and 0 at six, eight, 12 and 24 hours respectively.

In mice following subcutaneous administration of 100 mg./kg. twice daily, the blood serum contained 11 $\mu\text{g./cc.}$ and urine 60 $\mu\text{g./cc.}$ Guinea pigs receiving perorally by cannula 750 mg./kg. showed 2.3 $\mu\text{g./cc.}$ of serum at two hours and none at four hours, and when given the same amounts subcutaneously in propylene glycol, the serum contained 7.8 $\mu\text{g./cc.}$ at 30 minutes, 10.1, 3.9, 2.3, and 2.2 $\mu\text{g./cc.}$ at two, eight, 16 and 24 hours respectively, indicating that chloramphenicol orally in guinea pigs is either poorly absorbed or rapidly degraded in the body.

Urinary Excretion

Urine was collected from dogs by catheterization, and from small animals in a metabolism cage. Chloramphenicol concentration in urine attained maximum levels at two to eight hours, depending

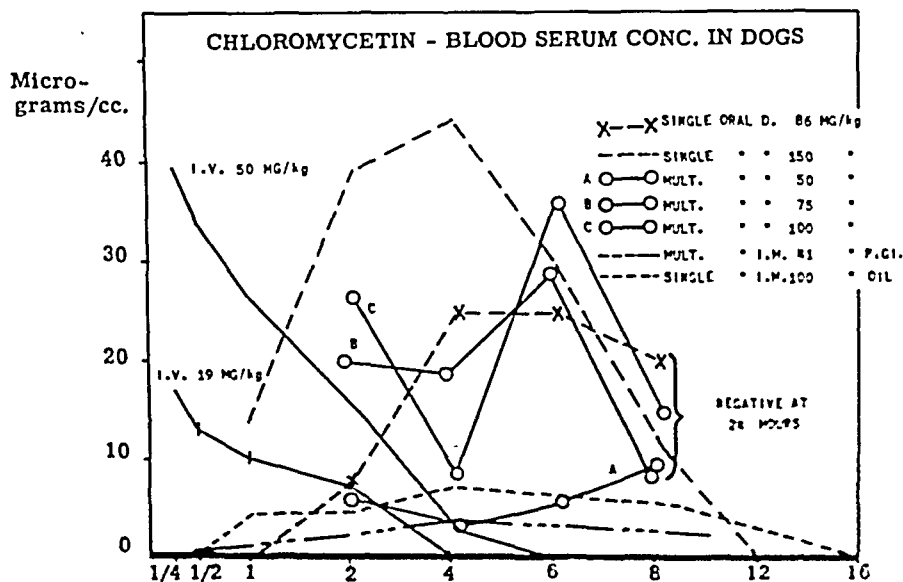


FIG. 4

Maximum serum levels occurred between two and six hours after oral administration and serum was negative in 12 to 24 hours. Chloramphenicol disappeared rapidly from serum on intravenous administration with negative findings at four to six hours.

upon mode of administration (Figure 5). Intravenous administration of single doses of 19 and 50 mg./kg. produced over 600 $\mu\text{g./cc.}$ of urine at two hours followed by gradual fall to 3 to 4 $\mu\text{g.}$ at 24 hours. Following oral single dose administration of 86 to 150 mg./kg. the urinary concentration reached a maximum of 1360 to 2500 $\mu\text{g./cc.}$ in four to six hours and 23 to 121 $\mu\text{g./cc.}$ at 24 hours. When given in oil suspension, a maximum concentration of 480 $\mu\text{g./cc.}$ was reached at eight hours and 7.5 $\mu\text{g./cc.}$ at 48 hours. Oral administration of two doses per day of 50 to 100 mg./kg. produced the first peak of 228 to 591 at two hours and the second peak at eight hours with 270

to 1300 $\mu\text{g./cc.}$, with 25 to 76 $\mu\text{g./cc.}$ at 24 hours. When given in propylene glycol, 41 mg./kg. twice daily intramuscularly, the two-hour averages for urinary concentration were 148 $\mu\text{g./cc.}$ and the 24-hour levels were 63 to 164 $\mu\text{g./cc.}$ (Table IV). The urinary excretion of chloramphenicol for a 24-hour period in dogs accounted for only 3.5 to 8.7 per cent of the total administered. Blood serum studies indicated absence of chloramphenicol in serum in four to six hours following intravenous and in 12 to 16 or 24 hours following oral or intramuscular administration, while urine contained considerable amounts at 24 hours, indicating absorption of Chloromycetin by tissues from

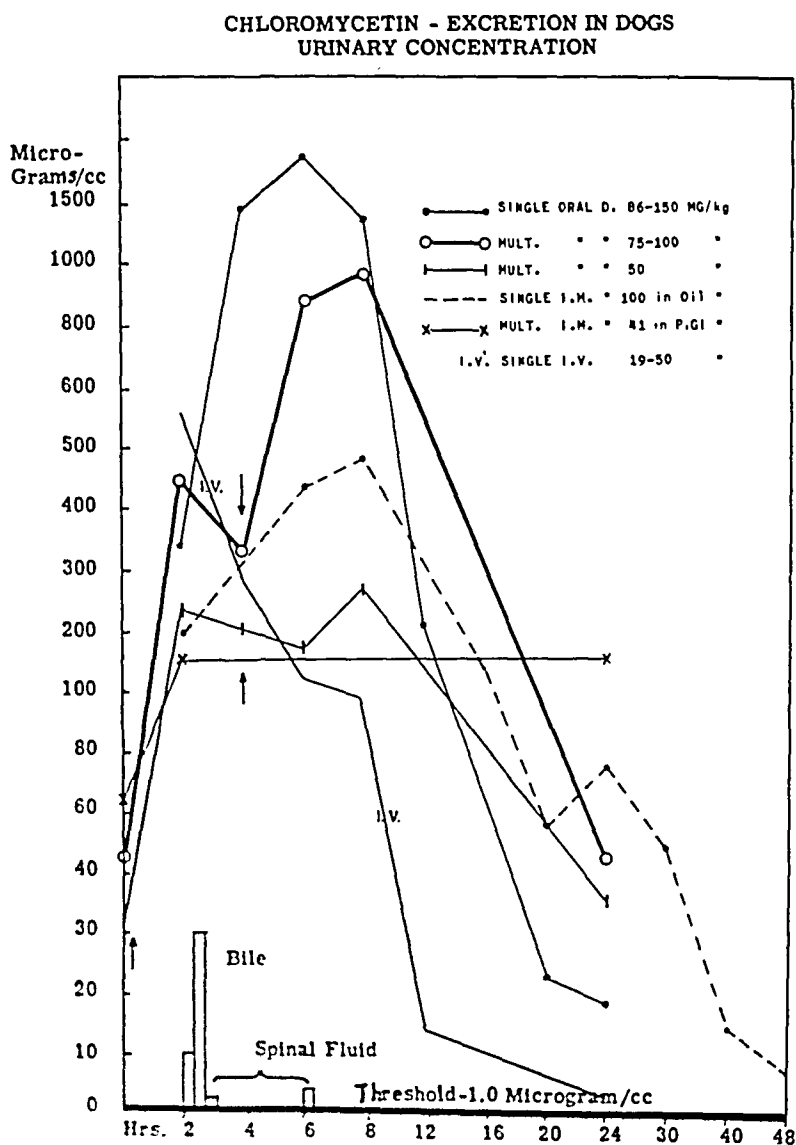


FIG. 5

High concentration of Chloromycetin occurred between two and 12 hours, peak at about eight hours, and the urinary excretion was prolonged over a 24-hour period.

TABLE IV
Urinary concentration of chloramphenicol in dogs

Number tests	Route	mg./kg. X daily	Hour intervals of sampling urine											
			0	2	4	6	8	12	16	20	24	30	40	48
			micrograms per cubic centimeter											
4	I.V.	19-50 X 1	0	485	137	47	23	14	—	—	3.3			
1	Oral	86 X 1	0	283	1360	2540	2080	—	—	—	121			
4	Oral	150 X 1	0	383	1487	1040	694	205	341	23	19.2			
6	Oral	50 X 2*	31	228	190	171	269	—	—	—	36			
20	Oral	75 X 2*	47	304	336	891	620	—	—	—	65			
6	Oral	100 X 2*	43	591	337	864	1300	—	—	—	25			
6	I.M. Oil	90-101 X 1	0	177	307	429	480	—	132	58	76	48	15	7.5
6	I.M. Pr. Gl.	41 X 2*	63	148	—	—	—	—	—	—	164			

* Second dose was administered at the end of the fourth hour.

which it is gradually liberated and excreted in the urine.

SUMMARY

1. Chloramphenicol (Chloromycetin) is moderately soluble (0.5 per cent) in water, urine and serum, propylene glycol (15 per cent), 50 per cent acetamide-water solution (5 per cent), and is insoluble in vegetable oils. Animal studies have indicated identical pharmacologic properties for material produced by fermentation and by chemical synthesis.

2. Chloramphenicol was readily absorbed following parenteral and oral administration and was found in blood serum, urine, bile and cerebrospinal fluid within two hours of administration.

3. It disappeared relatively rapidly from the blood stream, within four to six hours after intravenous and 12 to 16 hours after oral administration, with maximum blood serum concentration occurring between two and six hours and maximal urinary concentration between two and 12 hours. The urinary excretion persisted for over a 24-hour period with total recovery of 3.5 to 8.7 per cent.

4. Chloramphenicol was relatively non-toxic to animals and possessed no cumulative toxic effect on oral dosage of 100 to 200 mg. kg. per day to dogs for over a four-months period. The intravenous administration of 12.5, 25 and 50 mg. kg. was within the well-tolerated range. It caused no cumulative toxic effect on hemopoiesis, liver and kidney functions and visceral tissues. Blood pressure and respiration remained unaffected on

oral intake of 200 mg./kg. per day, and on intravenous administration to barbiturized dogs of 12.5 to 25 mg./kg. Gastro-intestinal reactions were uncommon, somatic reflexes, ocular and auditory functions remained undisturbed.

5. Lethal or near-lethal amounts of chloramphenicol orally and parenterally caused acute respiratory depression or failure, accompanied by fall of blood pressure and anoxia. Transient renal hematuria, probably of glomerular origin, and hydropic degeneration of glomerular capillaries occurred on administration of toxic amounts.

6. Intramuscular administration of oil suspensions or propylene glycol solution in doses of 0.5 to 1.0 gram caused considerable local tissue induration and necrosis, and a transient induration with 0.1 to 0.2 gram doses.

7. Oral administration of chloramphenicol to dogs provided measurable levels in the blood for about eight hours and in the urine for 24 hours.

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SUSCEPTIBILITY OF MICRO-ORGANISMS TO CHLORAMPHENICOL (CHLOROMYCETIN)^{1,2}

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The antibiotic, chloramphenicol, produced by *Streptomyces venezuelae*, was originally reported by Ehrlich *et al.* (1, 2) and independently described by Carter, Gottlieb *et al.* (3, 4). Since these original reports the active principle has been chemically isolated and characterized (5, 6), synthesized (7) and is at present commercially available for clinical use.

Originally (1) activity was described against several Gram-negative bacteria, in particular *Shigella sonnei*, and against *Rickettsia prowazekii* in chick embryos. Smadel *et al.* (8-11) have notably extended this spectrum to include other rickettsial agents as well as two viruses, lymphogranuloma venereum and psittacosis. Smith *et al.* (12) reported that chloramphenicol was active, under the conditions tested, against Gram-negative bacteria, *R. prowazekii* and *Borellia recurrentis*; moderately active against Gram-positive bacteria and *Mycobacterium tuberculosis*; and inactive in the concentrations used against yeasts, filamentous fungi, protozoa and certain viruses. Youmans *et al.* (13) tested strains of virulent human-type *M. tuberculosis in vitro* and concluded that chloramphenicol was only moderately active when compared with streptomycin or para-amino salicylic acid.

Clinically, confirmation of laboratory activity has been obtained for certain of the rickettsial diseases (14-18), typhoid fever (19), brucellosis (20) and in urinary infections caused by certain Gram-negative bacteria (21). With the drug now available in quantity for clinical trial, it seems opportune at this time to collect the known informa-

tion with respect to the microbiological spectrum of this new chemotherapeutic agent.

METHODS

Viruses and Rickettsiae

Borrelia: Vaccinia virus (New York Public Health strain from Dr. S. K. Muckenfus, New York City Dept. of Public Health, N. Y., egg-adapted) was tested as a representative of this group of viruses. Two types of tests in chick embryos were used. The first consisted of mixing the chloramphenicol with the virus and inoculating 0.1 ml. of the mixture onto the chorioallantoic membrane of 10-day chick embryos. After four days incubation at 35° C. the membranes were examined and compared with controls for reduction in pock formation. The second method consisted of inoculating groups of treated, 10-day chick embryos *via* the yolk sac with a lethal dose of virus and repeating treatment on the following day by the same route. The eggs were candled twice daily and delay in death time and seven-day survivors recorded.

Erro: St. Louis encephalitis virus (egg-adapted from Dr. Carl Duffy, Wayne University, Detroit, Michigan, and a mouse-adapted laboratory strain) was tested to represent the group of virus encephalitides. Tests were conducted in embryonated eggs and mice. For the egg test, nine-day embryos were infected *via* the allantoic sac and treated 30 minutes before infection and on the 1st day after infection. The eggs were candled twice daily and time of death and seven-day survivors noted. In the mouse test, groups of 10 to 12 Gm. white Swiss mice were infected intracerebrally and treated intraperitoneally before infection and twice daily thereafter for 10 doses. Mean death time was compared with that of a control group.

Formido: The National Institutes of Health mouse-adapted strain of rabies virus was used, as well as a variant line of this strain adapted to the intramuscular route of infection. For the former, mice were injected intracerebrally with a lethal dose and daily treatments given intraperitoneally for four days. A similar procedure was used with the intramuscular strain except for the different route of virus administration. Time of paralysis and death of the treated mice was noted for comparison with control groups.

Legion: The anti-poliomyelitis virus tests were conducted by Drs. J. L. Melnick and F. R. Coria (22) of the Yale University School of Medicine, New Haven, Conn. The Y-SK and Lansing strains (grouped together) were used as well as Theller's intestinal virus of mice.

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Rabula: The egg-adapted mumps virus tested was obtained from Dr. J. E. Smadel of the Army Medical School, Washington, D. C. The tests were conducted in seven-day embryonated eggs infected *via* the allantoic sac. Treatments were given 30 minutes before infection and on the second day. Allantoic fluid was harvested from each egg after seven-days incubation and tested for virus multiplication by the addition of washed chicken red blood cells. In addition a hemagglutination titration was performed on an aliquot pool of allantoic fluid from the groups of eggs. An untreated control group was included in the test for comparison of the number of positive eggs and level of group hemagglutinin titer.

Tarpeia: Tests against three strains of type A, seven strains of type A' and two strains of type B influenza virus have been conducted in 11-day chick embryos. The virus was inoculated into the allantoic sac and treatments given by the same route 30 minutes before infection and 60 minutes later. After 40 hours incubation at 35° C. the presence of infection in each egg and the hemagglutination titer of an aliquot pool of allantoic fluid were determined for each group as described above for mumps virus. Considerable work has been done using the mouse-adapted PR8 strain of type A in mice. In general, groups of 12 to 14 Gm. white Swiss mice were infected intranasally and treated by various routes and at different times with chloramphenicol. In the test reported here treatment was given intraperitoneally for seven days starting at the time of infection.

One inconclusive test has been made with the Green strain of distemper virus (commercial distemperoid, ferret-adapted) in ferrets. Two ferrets were infected intranasally and one was treated intraperitoneally at the time of infection and daily thereafter for three days. Severity of illness and time of development of fever were noted. The animals were sacrificed at the height of the disease; hence duration of illness could not be determined.

Tortor: Newcastle disease virus (California 14 from Dr. L. T. Giltner, Bureau of Animal Industry, Washington, D. C.) was tested in 11-day embryonated eggs and white Leghorn pullets. The test in eggs was conducted in the same manner as the influenza virus tests described above except that infection was determined by death or survival of the embryo. Comparisons of the average time of death of treated and untreated groups were made. In chickens the virus was administered intramuscularly and treatments were given approximately every 12 hours for four doses starting at the time of infection. Time of the appearance of symptoms and number of survivors compared with a control group were noted.

The chick bronchitis virus (obtained from Dr. C. H. Cunningham, Michigan State College, East Lansing, Michigan) was tested in chick embryos as described above for Newcastle disease.

Laryngo-tracheitis virus (commercial vaccine strain) was tested on the membrane of chick embryos using the technique described for vaccinia virus.

Rickettsia: Considerable work has been done with epidemic typhus (*R. prowazekii*, Breinl strain) in chick

embryos. In the work reported here, groups of six-day chick embryos were infected *via* the yolk sac and treated once on the third day after infection by the same route. Average time of death and number of survivors were compared with an untreated control group.

The results with murine typhus (*R. typhi*), scrub typhus (*R. tsutsugamushi*) and rickettsialpox (*R. akari*) were taken from the work of Smadel *et al.* (8-11). Using the method described above, their findings for murine typhus in eggs have been confirmed in this laboratory.

Dermacentroxenus, Coxiella and Miyagawanella: The reports of Smadel *et al.* (8-11) are included in the table for Rocky Mountain spotted fever (*D. rickettsi*), Q fever (*C. burnetii*), lymphogranuloma venereum (*M. lymphogranulomatis*) and psittacosis (*M. psittacii*).

Bacteria

The bacterial species reported here were tested for their susceptibility to chloramphenicol by inoculating a specified dilution or quantity of liquid culture into media containing varying concentrations of the drug, the end point being taken as the lowest concentration which caused complete inhibition of growth for 18 hours. Except as indicated the original transplants for preparation of suspensions were made from cultures dried from the frozen state.

The aerobes were tested by adding 1.0 ml. of a standardized dilution of a tryptose broth culture to 1.0 ml. quantities of tryptose broth containing varying concentration of chloramphenicol. The final concentrations used varied from 0.1 to 200 µg/ml. The tubes were incubated for 18 hours and the results recorded as "growth" or "no growth."

It was necessary to utilize special media for some of the more fastidious organisms. However, the same technique was used, replacing the tryptose broth with the special medium required. *Brucella abortus* was grown under 10 per cent CO₂ for 48 hours (No. 1335).

The anaerobes were tested by adding varying concentrations (ranging from 0.1 to 500 µg/ml.) of chloramphenicol to melted agar medium cooled to 40° C. To each tube, 0.1 ml. of a 24-hour liquid culture of the organism to be tested was added. The tubes were then shaken thoroughly and allowed to gel. Growth was recorded as positive or negative after 24 hours incubation.

Beef infusion agar (2 per cent) containing 2 per cent glucose (pH 8.2) was used for *Clostridium perfringens*, *novyi* and *septicum*. *Cl. fesceri* was tested in 2 per cent liver infusion agar (pH 8.2) and the anaerobic agar recommended by Records and Vawter (23) was utilized for *Cl. hemolyticum*. *Cl. tetani* was grown in Taylor medium containing 0.5 per cent agar and incubated in an atmosphere of hydrogen and carbon dioxide.

Fungi

In determining the susceptibility of fungi to chloramphenicol, a broth dilution method was employed. Serial dilutions of the drug in broth were dispensed in 1 ml. amounts in Wassermann tubes and to each tube was added

1 ml. of inoculum in broth. The tubes were incubated at 30° C. for one to seven days, depending upon the species, and then observed for inhibition of growth. The minimum concentration of the drug causing complete inhibition of growth was taken as the end point.

Modified Sabouraud's broth (dextrose 2 per cent, neo peptone 1 per cent) was used in preparing the dilutions of the drug and the fungus suspensions. In the case of the actinomycetes, Brain-Heart Infusion Broth (Difco) was employed.

Three types of inocula were used. 1) Spore suspension: In heavily sporulating species suspensions were prepared from either agar slant or agar plate cultures of the fungus. In the case of agar slants, broth was

added to the tube, the fungus stirred up with an inoculating needle and the tube then shaken. When using agar plate cultures the mycelium was scraped from the surface of the plate, placed in broth and shaken. The suspensions were then filtered through cotton and the spores counted on the hemocytometer. 2) Spores and mycelial fragments: Species having limited sporulation were removed from the surface of the culture medium, emulsified with broth either in a Waring Blender, by shaking with glass beads or by grinding in a mortar. The suspension was filtered through a loose cotton mat and in some instances the number of fragments and spores per ml. was determined by means of the hemocytometer. 3) Mycelial suspension: The mycelium of non-sporulating species was

TABLE I
Viruses and rickettsiae

Organism	Host	Route of infection*	Treatment (mg.)		Total dose	Result
			Schedule	Route		
Vaccinia	Emb.(10)†	C.A.M.	V+Cm. mixed	Membr.	0.05	Negative
Vaccinia	Emb.(10)	Y.S.	0.5×2	Y.S.	1.0	Negative
Variola (22)	Emb.	—	—	—	—	Negative
St. Louis Enceph.	Emb.(9)	Al.S.	0.5×2	Al.S.	1.0	Negative
St. Louis Enceph.	Mice	I.C.	0.3 q.d.×10	I.P.	3.0	Negative
Japanese Enceph.	Mice	—	—	—	—	Negative
Rabies	Mice	I.C.	0.5+0.2 q.d.×4	I.P.	1.3	Negative
Rabies	Mice	I.M.	0.25+1 q.d.×2	I.P.	2.25	Negative
Polio. (Lansing) (22)	Mice	—	—	—	—	Negative
Polio. (Y-SK) (22)	Mice	—	—	—	—	Negative
Theiler's Intestinal (22)	Mice	—	—	—	—	Negative
Mumps	Emb.(7)	Al.S.	0.5×2	Al.S.	1.0	Negative
Influenza						
A (PR8)	Mice	I.N.	2+1 q.d.×6	I.P.	8.0	Delayed death
A (3 strains)	Emb.(11)	Al.S.	0.25×2	Al.S.	0.5	Negative
A' (7 strains)	Emb.(11)	Al.S.	0.25×2	Al.S.	0.5	Negative
B (2 strains)	Emb.(11)	Al.S.	0.25×2	Al.S.	0.5	Negative
Distemper	Ferrets	I.P.	2+1 q.d.×3	I.P.	6.0	Negative
Newcastle Disease	Emb.(11)	Al.S.	0.3×2	Al.S.	0.6	Negative
Newcastle Disease	Chickens	I.M.	16.5 q.d.×12h×4	I.M.	66.0	6/8 Survivors
Chick Bronchitis	Emb.(11)	Al.S.	0.5×2	Al.S.	1.0	Negative
Laryngo-tracheitis	Emb.(10)	C.A.M.	V+Cm. mixed	Membr.	0.05	Negative
Lymphogranuloma (L.A.) (8-11)	Emb.(7)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
Psittacosis (6-BC) (8-11)	Emb.(7)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
Psittacosis (6-BC) (8-11)	Mice	I.P.	0.75 q.d.	I.P.	—	Significant
Psittacosis (P-4) (8-11)	Emb.(7)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
Epidemic Typhus	Emb.(6)	Y.S.	0.5×1 (3rd)	Y.S.	0.05	Significant
(Breinl) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
Murine Typhus						
(Wilmington) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
(Wilmington) (8-11)	Mice	I.P.	5.0 q.d.×13	Oral	65.0	Significant
Scrub Typhus						
(Gilliam) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.13	Significant
(Seerangayee) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.13	Significant
(Seerangayee) (8-11)	Mice	I.P.	2.5 q.d.×20	Oral	55	Significant
(Karp) (8-11)	Mice	I.P.	2.5 q.d.×12	I.P.	30	Significant
Rickettsialpox (MK) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.13	Significant
Rickettsialpox (MK) (8-11)	Mice	I.P.	0.75×10	Oral	7.5	Significant
R. M. spot. fever						
(Bitterroot) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.06	Significant
(Bitterroot) (8-11)	G. pigs	I.P.	50 q.d.	Oral	—	Doubtful
Q Fever (Henzerling) (8-11)	Emb.(6)	Y.S.	Pre-inf.	Y.S.	0.5	Significant

* C.A.M. = chorioallantoic membrane; Y.S. = yolk sac; Al.S. = allantoic sac; I.C. = intracerebral; I.M. = intramuscular; I.N. = intranasal; I.P. = intraperitoneal.

† Emb.(10) = chick embryos and (age of incubation).

TABLE II

Bacteria

Organism	P-D Culture Bureau No.	Source	Inoculum	Inhibiting conc. μg./ml.
<i>Aerobacter aerogenes</i>	0126	P. D. Stock	1-20,000,000	0.5
<i>Aerobacter aerogenes</i>	0126	Frequent transfer	1-10,000,000	2.5
<i>Alcaligenes faecalis</i>	01602	Pus	1-10,000,000	1
<i>Alcaligenes metalcaligenes</i>	—	Urine (B)	1-10,000,000	200
<i>Alcaligenes metalcaligenes</i>	—	Urine (B)	1-10,000,000	> 200
<i>Alcaligenes metalcaligenes</i>	—	Urine (B)	1-10,000,000	> 200
<i>Bacillus anthracis</i>	01172	B. A. I.	1:20	5
<i>Bacillus anthracis</i>	01176	B. A. I.	1:20	5
<i>Bacillus anthracis</i>	03191	College Station, Tex.	1:10	0.75
<i>Bacillus anthracis</i>	04809	A. T. C. C.	1:20	5
<i>Bacillus cereus</i> var. <i>mycoides</i>	04545	Frequent transfer	1-10,000,000	2.5
<i>Bacillus subtilis</i>	04771	Frequent transfer	1-10,000,000	2.5
<i>Brucella abortus</i>	1335	Dr. Huddleson, M. S. C.	1:20	10
<i>Brucella abortus</i>	02522	Aborted bovine fetus N. I. H.	1:20	2.5
<i>Brucella bronchiseptica</i>	03710	Trachea of dog	1:20	10
<i>Brucella bronchiseptica</i>	03854	Lung	1:20	10
<i>Brucella melitensis</i>	Gol.	Camp Detrick	1:20	5
<i>Brucella melitensis</i>	04091	N. I. H.	1:20	5
<i>Brucella suis</i>	1772	Dr. Huddleson, M. S. C.	1:20	5
<i>Clostridium fesceri</i>	01409	K. S. A. C.	0.1 cc. undiluted	> 500
<i>Clostridium fesceri</i>	01416	K. S. A. C.	0.1 cc. undiluted	> 500
<i>Clostridium hemolyticum</i>	04779	Univ. Nev.	0.1 cc. undiluted	> 500
<i>Clostridium hemolyticum</i>	04780	Univ. Nev.	0.1 cc. undiluted	> 500
<i>Clostridium novyi</i>	068	Dr. Novy, U. of M.	0.1 cc. undiluted	> 500
<i>Clostridium novyi</i>	04309	N. I. H.	0.1 cc. undiluted	> 500
<i>Clostridium perfringens</i> (welchii)	01400	Dr. DeKruif, U. of M.	0.1 cc. undiluted	> 500
<i>Clostridium perfringens</i> (welchii)	02122	Dr. Reed, Kingston, Ont.	0.1 cc. undiluted	> 500
<i>Clostridium septicum</i>	01587	Univ. of Calif.	0.1 cc. undiluted	> 500
<i>Clostridium septicum</i>	01759	K. S. A. C.	0.1 cc. undiluted	> 500
<i>Clostridium tetani</i>	03724	N. Y. Dept. of Health	0.1 cc. undiluted	> 500
<i>Clostridium tetani</i>	04770	Mueller variant	0.1 cc. undiluted	< 0.1
<i>Corynebacterium diphtheriae</i>	036	P. D. Stock	1-10,000,000	0.5
<i>Corynebacterium diphtheroides</i>	04464	P. D. Stock	1-10,000,000	1.0
<i>Corynebacterium pyogenes</i>	01536	Bovine uterus	1:10	2.5
<i>Diplococcus pneumoniae</i> Type I	04385	Purulent chest fluid	1-10,000,000	1.0
<i>Diplococcus pneumoniae</i> Type III	04645	P. D. Stock	1-10,000,000	2.5
<i>Diplococcus pneumoniae</i> Type XII	04338	N. I. H.	1-5,000,000	2.5
<i>Escherichia coli</i> var. <i>communior</i>	04420	Pyelitis, Rt. kidney	1-20,000,000	2.5
<i>Escherichia coli</i> var. <i>communior</i>	04256	Blood cult. uterine infection	1-20,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i>	04474	Kidney infection, urine	1-10,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i>	04508	Blood culture	1-10,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i>	04508	Blood culture	1-20,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i>	04746	Urine sp.	1-20,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i>	01495	Frequent transfer	1-10,000,000	2.5
<i>Escherichia coli</i> var. <i>communis</i> streptomycin-resistant	04814	P. D. Stock	1-10,000,000	0.5
<i>Escherichia coli</i> sp.	—	Urine (F)	1-10,000,000	2.5
<i>Escherichia coli</i> sp.	—	Urine (O)	1-10,000,000	2.5
<i>Escherichia coli</i> sp.	—	Pre-treatment sample of urine	1-10,000,000	2.5
<i>Gaffkya tetragena</i>	0584	Pure culture, U. of Iowa	1-10,000,000	2.5
<i>Hemophilus pertussis</i> (Phase I) (12)	04692	Dr. Kendrick, Mich.	10 ⁴ organisms	0.2
<i>Hemophilus pertussis</i> (Phase I) (12)	04688	Clinical case	10 ⁴ organisms	0.2
<i>Hemophilus pertussis</i> (5 isolates) (12)	—	Clinical cases	10 ⁴ organisms	0.2-0.3
<i>Klebsiella pneumoniae</i>	04012	Sputum	1-10,000,000	0.75
<i>Klebsiella pneumoniae</i>	04172	Blood culture	1-10,000,000	2.5
<i>Klebsiella pneumoniae</i>	04184	Spinal fluid	1-10,000,000	0.75
<i>Klebsiella pneumoniae</i>	04299	Sputum	1-10,000,000	0.5
<i>Klebsiella pneumoniae</i>	04389	Throat	1-10,000,000	0.5
<i>Klebsiella pneumoniae</i>	04483	Sputum	1-10,000,000	0.75
<i>Klebsiella pneumoniae</i>	04682	F. D. A.	1-500,000	0.5
<i>Klebsiella pneumoniae</i>	04682	F. D. A.	1-1,000,000	0.5
<i>Klebsiella pneumoniae</i>	04682	F. D. A.	1-10,000,000	0.5
<i>Klebsiella pneumoniae</i>	04682	F. D. A.	1-20,000,000	0.5
<i>Klebsiella pneumoniae</i> (smooth)	04812	F. D. A.	1-20,000,000	0.5
<i>Klebsiella pneumoniae</i> (opaque)	04812	F. D. A.	1-10,000,000	0.5
<i>Klebsiella pneumoniae</i>	04812	Frequent transfer	1-20,000,000	0.5
<i>Klebsiella pneumoniae</i>	04544	Frequent transfer	1-10,000,000	1.0
<i>Klebsiella pneumoniae</i> I	—	Urine	1-10,000,000	2.5

TABLE II—Continued

Organism	P-D Culture Bureau No.	Source	Inoculum	Inhibiting conc.
<i>Klebsiella pneumoniae</i> II	—	Urine	—	—
<i>Malleomyces mallei</i>	01828	P. D. Stock	1-5,000,000	15.0
<i>Micrococcus citreus</i>	0394	Boil on head	1:2000	37.5
<i>Micrococcus citreus</i>	03481	Gonorrhea	1-10,000,000	2.5
<i>Micrococcus pyogenes</i> var. <i>albus</i>	04460	Female gonorrhea	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>albus</i>	04475	Pus	1-10,000,000	2.5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04513	Pustule	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	Ear infection (M)	1-10,000,000	2.5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04816	F. D. A.	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04778	F. D. A.	1-5,000,000	2.5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04662	U. S. D. A.	1-10,000,000	1.0
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04598	U. S. D. A.	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04546	N. I. H.	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04540	F. D. A.	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04518	Blood culture	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04509	Boil	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	Ear (M)	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	—	Urine (Mc)	1-10,000,000	5
<i>Micrococcus pyogenes</i> var. <i>aureus</i>	04778	Frequent transfer	1-5,000,000	10
<i>Mycobacterium phlei</i>	—	Frequent transfer	1-5,000,000	5
(human virulent, 12 strains)	02482	Frequent transfer	1-10,000,000	5
<i>Neisseria catarrhalis</i>	02145	P. D. Stock	1-10,000,000	5
<i>Neisseria catarrhalis</i>	—	Fresh isolates	1-10,000,000	10
<i>Neisseria meningitidis</i>	—	Trachea at autopsy	—	25
<i>Pasteurella avicida</i>	03447	Tonsillar abscess	1-10,000,000	6.25
<i>Pasteurella avicida</i>	03995	Cough plate	1-5,000,000	to 12.5
<i>Pasteurella avicida</i>	04388	N. I. H.	1-5,000,000	0.5
<i>Pasteurella bollingeri</i>	04107	Heart blood of chicken	1-5,000,000	0.5
<i>Pasteurella bollingeri</i>	01673	Liver of goose	1-5,000,000	2.5
<i>Pasteurella bubalseptica</i>	04587	Heart blood of chicken	1:2000	2.5
<i>Pasteurella cuniculicida</i>	04588	Colorado Agric. Coll.	1:2000	0.5
<i>Pasteurella oviseptica</i>	02855	Calf's lung	1:2000	0.25
<i>Proteus vulgaris</i>	04042	Buffalo strain	1:2000	0.25
<i>Proteus vulgaris</i>	02217	Sinus of rabbit	1:10	0.25
<i>Proteus vulgaris</i>	01345	Colorado Agric. Coll.	1:2000	0.75
<i>Proteus vulgaris</i>	02854	P. D. Stock	1:2000	0.5
<i>Proteus vulgaris</i>	0924	N. I. H.	1:2000	0.5
<i>Proteus vulgaris</i>	02164	N. I. H.	1-10,000,000	2.5
<i>Proteus species No. 1</i>	02165	N. I. H.	1-10,000,000	2.5
<i>Proteus species No. 2</i>	04262	N. I. H.	1-10,000,000	5
<i>Proteus species No. 3</i>	04736	Urine	1-10,000,000	2.5
<i>Proteus species No. 4</i>	04736	Frequent transfer	1-10,000,000	1.0
<i>Pseudomonas aeruginosa</i>	—	—	1-10,000,000	2.5
<i>Pseudomonas aeruginosa</i>	—	—	1-10,000,000	2.5
<i>Pseudomonas aeruginosa</i>	—	—	1-10,000,000	2.5
<i>Pseudomonas aeruginosa</i>	04126	Urine	1-10,000,000	25
<i>Pseudomonas aeruginosa</i>	04248	Eye	1-10,000,000	10
<i>Pseudomonas aeruginosa</i>	02133	Swab from left arm	1-10,000,000	5
<i>Pseudomonas aeruginosa</i>	04591	Horse	1-10,000,000	25
<i>Pseudomonas aeruginosa</i>	04625	Abdominal cavity of chicken	1-10,000,000	50
<i>Pseudomonas aeruginosa</i>	04629	Turkey	1-10,000,000	37.5
<i>Pseudomonas aeruginosa</i>	2430	Bovine	1-10,000,000	75
<i>Pseudomonas aeruginosa</i>	01925	Frequent transfer	1-10,000,000	75
<i>Pseudomonas aeruginosa</i>	01925	Stool culture	1-10,000,000	50
<i>Pseudomonas aeruginosa</i>	01926	Frequent transfer	1-10,000,000	>200
<i>Pseudomonas aeruginosa</i>	01931	Eye	1-10,000,000	37.5
<i>Pseudomonas aeruginosa</i>	04728	Stool culture	1-10,000,000	37.5
<i>Pseudomonas aeruginosa</i>	—	Urine	1-10,000,000	37.5
<i>Pseudomonas aeruginosa</i> (rough type)	—	Urine (Mc)	1-10,000,000	25
<i>Pseudomonas aeruginosa</i> (smooth type)	—	Urine (C)	1-10,000,000	25
<i>Pseudomonas aeruginosa</i>	—	Urine (Mc)	1-5,000,000	100
<i>Rhodospirillum rubrum</i>	—	Urine (Mel)	1-10,000,000	50
<i>Salmonella enteritidis</i>	—	Urine (Mel)	1-10,000,000	50
<i>Salmonella enteritidis</i>	—	Urine (S)	1-5,000,000	15
<i>Salmonella gallinarum</i>	01348	Urine (L)	1-10,000,000	50
—	0013	P. D. Stock	1-10,000,000	15
—	01792	P. D. Stock	1-5,000,000	10
—	04564	P. D. Stock	1-10,000,000	25
—	—	Chicken origin	1-10,000,000	0.25
—	—	—	1-10,000,000	0.75
—	—	—	1-10,000,000	2.5
—	—	—	1-10,000,000	2.5

processed in the manner described for preparing the suspension of spores and mycelial fragments.

All of the fungi were tested first in a series of dilutions ranging from 50 to 1000 µg/ml. of the drug. The actinomycetes, *Actinomyces bovis* and *Nocardia asteroides*, were retested at lower concentrations and four species, *Trichophyton interdigitale*, *T. mentagrophytes*, *T. rubrum* and *Microsporum canis*, at higher concentrations (up to 2500 µg/ml.) of chloramphenicol. In order to keep the drug in solution at the higher concentrations 1.8 ml. of the drug-broth mixture and 0.2 ml. of inoculum were used in each tube instead of 1 ml. of each as in the lower concentration series.

Spirochetes and Protozoa

The authors are indebted to Dr. Paul E. Thompson (24) and Dr. O. M. Gruhitz (25) of the Parke, Davis Research Laboratories for the data included in this section.

In Vitro Tests: *Endamoeba histolytica* (University of Chicago strain, with mixed bacterial flora) was tested in two media, Egg-Locke diphasic and Balamuth (essentially protein free). Various concentrations of chloramphenicol were incorporated in these media and amebicidal activity determined by microscopic examination for motility after 24 hours at 37° C.

Borrelia novyi and *recurrentis* and *Trichomonas foetus*

TABLE III
Fungi

Fungus	Bureau No.	Source	Inoculum		Inhibition µg./ml.
			Type	No./ml.	
<i>Actinomyces bovis</i>	04	P. D. Stock	Mycel. Susp.*	—	5
<i>Allernaria</i> sp.	04738	P. D. Stock	Spores	281,000	>1000
<i>Allescheria boydii</i>	04619	N. I. H.	Spores	500,000	>1000
<i>Aspergillus clavatus</i>	04445	Dr. Waksman	Spores	500,000	>1000
<i>Aspergillus flavus</i>	04456	Dr. Thom	Spores	500,000	>1000
<i>Aspergillus fumigatus</i>	04612	N. I. H.	Spores	500,000	>1000
<i>Aspergillus glaucus</i>	04821	P. D. Stock	Spores	500,000	>1000
<i>Aspergillus nidulans</i>	04434	P. D. Stock	Spores	500,000	>1000
<i>Aspergillus niger</i>	01383	P. D. Stock	Spores	500,000	>1000
<i>Blastomyces dermatitidis</i>					
Mycelial phase	04613	N. I. H.	Spores & Mycel.†	500,000	>1000
<i>Candida</i> (Monilia) <i>albicans</i>	04600	Bronchus	Spores	500,000	>1000
<i>Cryptococcus neoformans</i>					
(Torula histolytica)	04611	N. I. H.	Spores	500,000	>1000
(Torula histolytica)	04817	Meningitis	Spores	500,000	>1000
<i>Epidermophyton floccosum</i>	04624	Dr. Thompson	Spores & Mycel.†	375,000	>1000
<i>Helminthosporium sativum</i>	04677	Tinea cruris	Spores	250	>1000
<i>Histoplasma capsulatum</i>					
Mycelial phase	04799	A. T. C. C.	Spores & Mycel.†	—	>1000
Mycelial phase	04800	N. I. H.	Spores & Mycel.	—	>1000
<i>Hormodendrum</i> sp.	04637	Dr. Downing	Spores	500,000	>1000
<i>Microsporum audouinii</i>	04606	N. I. H.	Mycel. Susp.‡	20,000	>1000
<i>Microsporum canis</i>	04605	N. I. H.	Spores & Mycel.†	100,000	>2500
<i>Microsporum gypseum</i>	04604	N. I. H.	Spores	125,000	>1000
<i>Mucor racemosus</i>	01385	P. D. Stock	Spores	500,000	>1000
<i>Nocardia asteroides</i>	04618	N. I. H.	Mycel. Susp.*	—	20
<i>Penicillium expansum</i>	01386	P. D. Stock	Spores	500,000	>1000
<i>Phialophora verrucosa</i>	04617	N. I. H.	Mycel. Susp.*	—	>1000
<i>Rhizopus nigricans</i>	04529	A. T. C. C.	Spores	500,000	>1000
<i>Rhodotorula</i> sp.	01527	P. D. Stock	Spores	500,000	>1000
<i>Saccharomyces carlsbergensis</i>	04450	Fleischmann	Spores	500,000	>1000
<i>Saccharomyces cerevisiae</i>	01525	P. D. Stock	Spores	500,000	>1000
<i>Saccharomyces ellipsoideus</i>	01524	Mich. State Coll.	Spores	500,000	>1000
<i>Sporotrichum schenckii</i>	04614	N. I. H.	Spores	500,000	>1000
<i>Trichophyton acuminatum</i>	04607	N. I. H.	Spores	485,000	>1000
<i>Trichophyton interdigitale</i>	04452	N. I. H.	Spores	500,000	>2500
<i>Trichophyton mentagrophytes</i>	04781	N. I. H.	Spores	500,000	>2500
<i>Trichophyton rubrum</i> (T. <i>purpureum</i>)	04639	N. I. H.	Spores	500,000	>2500
<i>Trichophyton sabouraudi</i>	04638	N. I. H.	Spores & Mycel.‡	—	>1000
<i>Trichophyton schoenleinii</i>	04603	N. I. H.	Mycel. Susp.*	—	>1000
<i>Trichophyton sulfurum</i>	04608	N. I. H.	Spores	500,000	>1000
<i>Trichophyton tonsurans</i>	04609	N. I. H.	Spores	730,000	>1000
<i>Trichophyton violaceum</i>	04676	A. T. C. C.	Mycel. Susp.*	—	>1000

* Suspension of emulsified mycelial material. The number of fragments was not determined.

† Both spores and mycelial fragments are included in the count.

‡ Light suspension of spores and mycelial fragments.

§ Mycelial fragments counted.

were suspended in a suitable menstruum containing varying concentrations of chloramphenicol, as indicated in Table IV, and observed for immobilization. Observations were made after two hours in the case of the *Borreliae* and after seven hours exposure for trichomonas.

In Vivo Tests: *Plasmodium lophurae* was tested in ducks (intraperitoneal treatment) and chicks (oral treatment) and the results compared with the activity of quinine.

Rats and dogs were used to evaluate the activity of chloramphenicol against *E. histolytica* *in vivo*. Treatment was given orally and results expressed as number of animals cleared of infection or degree of suppression of infection.

B. novyi was tested in groups of mice treated by either the intraperitoneal or oral route. Results are expressed as percentage suppression of spirochetemia.

Rabbits infected with *Treponema pallidum* were treated with various concentrations of chloramphenicol twice daily for eight days. Rate of disappearance of treponemes and degree of healing of lesions under treatment were noted. The animals were held to check for relapses in the dosages at which healing under treatment was noted.

RESULTS

Tables I to IV summarize the results obtained with the different micro-organisms that have been tested in the laboratory. Wherever possible the minimal inhibiting concentration of chloramphenicol for the strain, under the conditions tested, is given. Otherwise, the maximum concentration tested or the dose administered, in the case of *in vivo* tests, is entered. The identifying numbers given for the bacteria and fungi are those assigned by the Parke, Davis and Company culture bureau, where a complete history of the strain is available. Strains without assigned numbers are for the most part recent isolates from clinical cases prior to or during treatment with chloramphenicol.

The Emergence of Microbial Resistance

With the finding of considerable variation in susceptibility to chloramphenicol of different cultural lines of certain bacterial species, it became

TABLE IV
Spirochetes and protozoa

In vitro tests					
Organism	Medium or menstruum	Time	Conc.	Result	
			µg./ml.		
<i>Endamoeba histolytica</i> *	L. E. L.	24 hours	1000	Negative	
<i>Endamoeba histolytica</i> *	Balamuth	24 hours	250	Significant	
<i>Borrelia novyi</i> *	50% horse serum	2 hours	10 to 50	Immobilization	
<i>Borrelia recurrentis</i> *	2.5% rat serum	2 hours	2.5	Immobilization	
<i>Trichomonas foetus</i> †	0.7% sodium chloride	7 hours	2000	Negative	
<i>Pelomyxa carolinensis</i> †	Pace & Kimura buffer	48 hours	2500	Negative	
<i>Tetrahymena geleii</i> †	2% proteose peptone	48 hours	2500	Negative	
In vivo tests					
Organism	Host	Treatment			Result
		Dose	Schedule	Route	
		mg./kg./day	days		
<i>Plasmodium lophurae</i> *	Ducks	200	b.d.×5	I.P.	Q = <0.05
<i>Plasmodium lophurae</i> *	Chicks	537	5	Diet	Q = <0.1
<i>Endamoeba histolytica</i> *	Rats	583-868	7	Diet	9/14 cleared
<i>Endamoeba histolytica</i> *	Dogs	200	b.d.×10	Oral	Suppression
<i>Borrelia novyi</i> *	Mice	14.9	5	Diet	50% Suppression
<i>Borrelia novyi</i> *	Mice	7.6	×3(4, 22, 28 hrs.)	I.P.	50% Suppression
<i>Treponema pallidum</i> §	Rabbits	25	b.d.×8	I.M.	Negative
<i>Treponema pallidum</i> §	Rabbits	50-100	b.d.×8	I.M.	Spirochetes and lesions cleared 4-5 days, relapsed

* Thompson, P. E., Research Laboratories of Parke, Davis & Company, personal communication and (12).

† Reutner, T. F., Research Laboratories of Parke, Davis & Company and (12).

‡ Pace, D. M., and Russell, D., quoted (12).

§ Gruhitz, O. M., personal communication, Research Laboratories of Parke, Davis & Company.

|| Quinine equivalent.

TABLE V
Induced resistance to chloramphenicol in bacteria

Organism	P-D Culture Bureau No.	Transfers on chloramphenicol containing medium	Inhibiting conc. chloramphenicol µg./ml.	
			Original	Final
<i>Salmonella typhosa</i>	clinical case	12	10	250
<i>Aerobacter aerogenes</i>	0126	12	5	50
<i>Escherichia coli</i>	01495	12	10	500
<i>Proteus</i> (sp.)	04736	8	500	>4000
<i>Pseudomonas aeruginosa</i>	01925	8	1250	>4000
<i>Klebsiella pneumoniae</i>	04544	12	5	15
<i>Alcaligenes metalcaligenes</i>	clinical case	7	1250	2250

of interest to determine whether increased resistance to chloramphenicol could be induced by laboratory manipulation. Our early observations indicate that certain species of micro-organisms will develop resistance to chloramphenicol when sub-inoculated on media containing increasing concentrations of the drug. The bacterial species tested include *Salmonella typhosa*, *Aerobacter aerogenes*, *Escherichia coli*, *Proteus* sp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Alcaligenes metalcaligenes*.

The test was carried out by sub-inoculating 0.1 ml. of the various cultures into Brain-Heart Infusion Broth (Difco) with 10 per cent horse serum added and containing various concentrations of chloramphenicol. The cultures were incubated for 72-96 hours. From the tube containing the highest concentration of chloramphenicol, in which good growth was obtained, 0.1 ml. was sub-inoculated into 2.0 ml. of medium containing the same and higher concentrations of the drug. In Table V the preliminary results of this study are indicated. It can be seen that the susceptibility of these organisms has decreased from approximately 2 to 50 fold in the course of these passages.

The results of tests designed to detect a similar phenomenon for *Rickettsia prowazekii* have so far been negative. One series has been carried through 13 passages, and a second through six passages in chick embryos treated with partially inhibiting doses of chloramphenicol. Comparative tests of the passaged and stock strains have indicated no change in susceptibility to the drug under these conditions.

SUMMARY AND CONCLUSIONS

Sixty-four genera of micro-organisms including 290 species and strains have been tested for their susceptibility to chloramphenicol. The results are recorded in Tables I to IV.

From Table I it appears that all of the *Rickettsiae* tested are susceptible to chloramphenicol. *Coxiella burnetii* (Q Fever) may be more resistant than the others as larger doses are required to produce corresponding delay in mean death time of treated embryos. Possibly the whole lymphogranuloma venereum-psittacosis group of virus (*Miyagawanella*) will prove to be as susceptible as the type species. There are indications that influenza virus in mice and the virus of Newcastle disease of chickens respond somewhat to comparatively massive doses of the drug. It is possible, however, that the therapeutic effect seen can be explained by the action of the drug upon secondary bacterial invaders. While this hypothesis is apparently borne out by the negative results with chloramphenicol on these viruses in embryonated eggs, further work and possibly controlled clinical trials are indicated.

Tests against the virus of poliomyelitis have proved negative. Dr. J. L. Melnick states as follows: "I have gone over the records of the work done with Dr. Filiberto Ramirez Corria on the use of this material on two strains (Lansing and Y-Sk) of poliomyelitis virus and on Theiler's intestinal virus of mice. When the viruses were titrated using saturated aqueous solutions of Chloromycetin as diluent, there was no effect on the titer even when the viruses were inoculated into mice receiving daily inoculations of the drug (1 cc. of saturated solution intraperitoneally). Further-

more, daily oral administration of Chloromycetin did not appear to have any effect on the intestinal carrier state⁴ of mice spontaneously harboring Theiler's virus of mouse encephalomyelitis" (22).

From Table II, which gives the results from tests of over 200 bacterial strains representing 25 genera, it can be concluded that considerable variation in susceptibility is encountered between genera as well as between species and even strains within the species. Under the conditions tested susceptibility to concentrations of 10 $\mu\text{g}/\text{ml}$. or less, indicating possible clinical application, was observed for 21 of the genera. These include *Aerobacter* (2/2),⁴ *Bacillus* (6/6), *Brucella* (7/7), *Corynebacterium* (3/3), *Diplococcus* (3/3), *Escherichia* (11/11), *Hemophilus* (7/7), *Klebsiella* (11/12), *Micrococcus* (19/19), *Neisseria* (4/4), *Pasteurella* (8/8), *Proteus* (8/10), *Pseudomonas* (1/19), *Salmonella* (37/37), *Sarcina* (3/3), *Shigella* (16/16), *Streptococcus* (8/8) and *Vibrio* (1/1). *Pseudomonas*, though fairly resistant, is included since it seems to be susceptible to chloramphenicol in concentrations obtainable in the urine. The results given for *Alcaligenes* leave the status of this genus somewhat in doubt. A stock strain of *A. faecalis* was found to be susceptible but three different isolates from the same clinical case of *A. metalcaligenes* infection were found to be highly resistant. A somewhat similar situation is observed for *Proteus vulgaris* where two of ten strains tested required a somewhat higher concentration (25 $\mu\text{g}/\text{ml}$.) for complete inhibition. An interesting finding was encountered with the *Clostridia*. While this group in general was of very low susceptibility, one strain, the Mueller variant of *Cl. tetani*, was very susceptible. This variant does not produce spores in culture, which raises the speculation that possibly the apparent resistance of this group is a reflection of the resistance of the spores rather than of the vegetative forms.

While not within the scope of this paper, *in vivo* laboratory trials with certain of the bacterial agents have been conducted. Gould *et al.* (26) report encouraging results with the cholera vibrio in mice and Gruhzit (25) has found activity in mice

against certain of the Gram-positive cocci and the salmonella group. Sarber (27) has treated mice infected with *Hemophilus pertussis* and found chloramphenicol to be an effective chemotherapeutic agent. Clinical trials are in progress against many of these pathogens. To date clinical confirmation has been obtained in typhoid fever (19), brucellosis (20), and certain urinary infections caused by Gram-negative species (21).

Of the forty species or strains of fungi tested for susceptibility to chloramphenicol, Table III, only two were completely inhibited by the drug at the concentrations used in the test. These two species, *Actinomyces bovis* and *Nocardia asteroides*, both of which belong to the actinomycetes, were inhibited by 5 $\mu\text{g}/\text{ml}$. and 20 $\mu\text{g}/\text{ml}$. of the drug, respectively. Since blood levels of this magnitude are easily attained, trial on clinical infections with these agents is indicated.

Growth of four species of ringworm fungi, *Trichophyton mentagrophytes*, *T. interdigitale*, *T. rubrum* and *Microsporum canis*, was retarded by 1000 $\mu\text{g}/\text{ml}$. of the drug but complete inhibition could not be obtained even when the drug concentration was increased to 2500 $\mu\text{g}/\text{ml}$.

With the possible exception of the spirochete of relapsing fever (*Borrelia recurrentis*), the results with the spirochetes and protozoa tested were not promising. Doses of 12.5 mg/Kg. twice daily for eight days were ineffective on syphilitic lesions in rabbits. Although similar treatment with 25 or 50 mg/Kg. cleared the lesions, relapses occurred when treatment was stopped.

Attempts to induce increased resistance to chloramphenicol by passage in the presence of the drug have been successful in the case of seven bacterial species, but not for *R. prowazekii* in the embryonated egg. It remains to be seen whether, under practical conditions in the clinic, this finding will be of importance.

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⁴ Indicates number susceptible strains/number of strains tested.

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PRELIMINARY REPORT ON THE USE OF CHLORAMPHENICOL (CHLOROMYCETIN) IN THE TREATMENT OF ACUTE GONORRHEAL URETHRITIS¹

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The great advances which have been made in recent years in the treatment of gonorrheal infections would appear to have provided the medical profession with adequate therapeutic agents for this disease. The numerous advantages of penicillin treatment have resulted in its replacing the sulfonamide drugs, except in selected cases (1). Even the disadvantages of parenteral injections of penicillin have been avoided by a number of workers who have administered the antibiotic by mouth (1). Indeed, Campbell, Dougherty and Curtis reported on the oral use of penicillin in the prophylaxis of gonorrhea at an earlier session of this Symposium (2). Despite the fact that penicillin has many attributes of an ideal drug for the treatment of gonorrhea, it has one distinct drawback, *i.e.*, its suppressive effect on a coexisting syphilitic infection. This problem of the possible masking of syphilis by treating acute gonorrhea patients with amounts of penicillin which are adequate for the bacterial infection but inadequate for the spirochetal infection is of tremendous importance (1, 3). Therefore, the search must continue for a drug with the activity of penicillin against the gonococcus but without its marked anti-spirochetal action. This report is a progress note which presents certain preliminary data suggesting that chloramphenicol, or Chloromycetin,² approaches penicillin in its efficacy in the treatment of acute gonorrheal urethritis in the male and that this new drug is distinctly less spirocheticidal than penicillin.

During the past three months in Malaya we have used Chloromycetin in treating 45 men with gonorrhea. Thirty-four of these received the synthetic type of drug and 11 the fermentation type. Data on 32 patients in this series are summarized in Table I. Each person had a purulent urethral discharge when admitted to the study. This had been present for three days or less in the majority of instances, but three individuals in the group had suffered for 21, 28, and 60 days, respectively. The mean duration of disease when therapy was begun was from four to 12 days in the four subgroups listed in Table I. Smears of the exudate taken from each patient

TABLE I
Chloromycetin in the treatment of 32 males with acute gonorrheal urethritis

	Chloromycetin dosage			
	Synthetic		Fermentation	
	3.0-3.5 grams	1.0-1.5 grams	3.0-3.5 grams	1.0-1.5 grams
Number of cases	11	10	6	5
Day of illness treatment begun				
Mean	12	4	8	4
Range	1-60	1-10	1-21	1-10
Urethral discharge				
Hours duration post-treatment				
Mean	43	43	53	42
Range	5-72	24-72	7-96	18-60

on admission contained numerous Gram-negative intracellular diplococci. Furthermore, cultures, made on Harrison's agar, yielded *N. gonococci* from all but one of the cases; the single exception suffered a relapse on the eighth day and is included because of this.

Each of the patients was treated with a single oral dose of one or the other type of Chloromycetin and permitted to continue his normal occupational duty. Slightly more than half the patients listed in the table received a total of 3.0 or 3.5 grams and the remainder were given 1.0 or 1.5 grams. It is apparent from the tabular data that similar results were obtained as regards disappearance of urethral discharge in the four subgroups, *i.e.*, patients who received the smaller or larger dose of either the synthetic or fermentation type of drug. Later on

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² Trade name of Parke, Davis and Company. The Chloromycetin used in the present work was supplied by Parke, Davis and Company. Lots of drug produced by the fermentation and by the synthetic process were made available.

additional comments will be made on other patients not mentioned in the table who were treated with the smaller doses of drug.

Within 24 hours after the therapeutic dose had been given the urethral discharge had disappeared in 13 of the 32 patients. Furthermore, in another 17 of the group the discharge was reduced to a drop or so of thin watery material which was obtained by stripping. Two of the 32 persons still yielded a drop of purulent exudate at this time. Stained smears of the 19 exudates revealed that only one had Gram-negative intracellular diplococci; the culture of this material failed to yield *N. gonococci*. However, a few colonies of *N. gonococci* were grown from a thin milky exudate of another patient who was cultured 24 hours after being given 3.0 grams of drug. Furthermore, the material from three other patients taken at this time yielded colonies resembling *N. gonococci*.

On the third to fifth days after treatment was given, 30 of the 32 patients were subjected to prostatic massage. Materials for smear and culture were obtained from 18 of these. In no instance were Gram-negative intracellular diplococci found microscopically; indeed, even leukocytes were recorded as being absent from the smears of 12 of the specimens. Seventeen of the 18 cultures of prostatic secretion taken during this period contained no *N. gonococci* but from one secretion a few colonies were grown which resembled gonococci morphologically. Three subsequent massages of Patient 4, who had supplied this questionable material, were non-productive and he has now remained well for eight weeks.

A total of four relapses have been noted to date among the 32 patients who have been tabulated. One occurred 28 days after treatment with 3.0 grams of synthetic drug, which was 26 days after complete disappearance of all signs of disease. This individual had been re-exposed recently and he may represent a reinfection. Two patients relapsed on the 19th and eighth days, respectively, after treatment with 3.0 grams of synthetic drug; discharges of both had initially cleared within 48 hours and recurred following inebriation. The fourth case was apparently cured within 36 hours after receiving 1.0 gram of fermentation drug but relapsed on the 14th day. Each of these four patients was again treated with a single dose of 3.0 grams of Chloromycetin and responded well.

Three additional patients not included in the table are worthy of mention. Each received a single dose of 1.0 or 1.5 grams of Chloromycetin but was subsequently given an additional 3.0-gram dose one to four days later. This additional medication appeared indicated because of the persistence of some discharge, or the presence of Gram-negative intracellular diplococci. However, none of the cultures taken at the time the supplementary drug was administered were later found to contain gonococci. Each of the three patients recovered promptly after being given the additional drug. Thus, it appears that the administration of 1.0 gram or 1.5 grams of drug in a single dose may provide inadequate therapy in a proportion of cases.

Smith and his coworkers (4) found that rabbits which had been infected with the Nichols strain of *Treponema pallidum* and then treated with Chloromycetin for eight days at the rate of 25 mg. per kilo per day showed no change in their lesions nor did the spirochetes disappear. They also noted that daily doses of 50 and 100 mg. per kilo cleared the lesions of spirochetes but that the effect was temporary.

Our experience with Chloromycetin in two patients with primary chancres suggests that the results in man may be similar to those just mentioned in rabbits. Dark field examination of fluid from the lesion of one man showed an average of three motile spirochetes per field. The patient received 4.0 grams of Chloromycetin in a single dose (45 mg. per kilo of body weight) and repeated examinations over the next 18 hours revealed the same number of active treponema. He was then given 3.0 grams of drug at one time followed by five doses of 0.5 gram each at three-hour intervals, thus totaling 5.5 grams (62 mg. per kilo) over this 18-hour period. At the end of this time two spirochetes of reduced motility were found in the three preparations examined and eight hours later only one non-motile organism was seen in two samples. During the succeeding week the chancre healed slowly and spirochetes were not observed. The second patient was given an initial dose of 70 mg. per kilo of body weight and an additional 58 mg. per kilo over the next 15 hours. Seventeen hours after treatment was started motile spirochetes could no longer be found but there was

no evidence of healing of the chancre during the succeeding three days.

CONCLUSIONS

The present information, although preliminary, indicates that Chloromycetin is of some value in the treatment of acute gonorrheal urethritis in the male. Single oral doses have been employed almost exclusively in these studies; the efficacy of the same total amounts of drug given in divided doses over a period of one or two days should be investigated.

Chloromycetin is appreciably less effective than penicillin against *Treponema pallidum*. Since an agent capable of controlling gonorrhea without masking syphilitic infection is to be desired, further studies of the use of Chloromycetin in the treatment of venereal disease are indicated.

ADDENDUM

Since the above was submitted additional cases have been added to this series and supplementary data obtained on the original patients. One lot of chloramphenicol, presumed to be fermentation type, later proved to be synthetic; this necessitated minor changes in the tabulation of the data. A total of 48 patients with acute gonorrheal urethritis have been treated with chloramphenicol. Of these, 42 were given synthetic drug in a single oral dose. Twenty-four were given 3.0 to 3.5 grams and 18 were given 1.0 to 1.5 grams. The remaining six received 3.0 to 3.5 grams of fermentation drug. The mean duration of disease in these three sub-groups prior to treatment was 8, 4, and 11 days, respectively. The clinical response was essentially the same in each sub-group; therefore, neither the type of drug nor the dosage employed appeared to affect the results. Dysuria disappeared within 36 hours (average for the 48 patients) and discharge (as determined by the absence of exudate on stripping) within 49 hours. Smears of the discharge with few exceptions were free of gonococci by the next day and leukocytes generally were absent from the discharge in those instances where this sign persisted longer than 48 hours. Ten patients or 20 per cent of this entire series had relapses during the month following treatment. There was no apparent difference in the number of recur-

rences in the three sub-groups. All relapses were treated with a single oral 3.0 gram dose of chloramphenicol and responded satisfactorily.

The primary lesion of the first patient with a syphilitic chancre mentioned in the text, who received 9.5 grams of chloramphenicol over a two-day period, healed completely by the tenth day after beginning treatment. During the fourth week a chancre containing spirochetes reappeared at the site of the original lesion. The patient was then retreated, receiving three single 1.0 gram oral doses of synthetic drug at daily intervals. Spirochetes disappeared from the recurrent chancre the day following the third dose of drug and the lesion was completely healed nine days later. On the 44th day after the first appearance of the chancre and the 36th day after initial treatment, a Kahn test gave a plus-minus reaction; it had been consistently negative previously. The three chancres of the second patient, who received 5.5 grams during 18 hours, healed by the 12th day but 30 days later a fresh chancre reappeared at one of the original sites. The Kahn reaction of the second patient remained negative throughout the 35 days of observation following the original appearance of the lesions.

Nine patients with acute gonorrheal urethritis were treated with single oral doses of 3.0 to 4.25 grams of aureomycin. In this small series the clinical effect was essentially similar to that obtained with chloramphenicol and the results were somewhat better than those obtained by Collins *et al.* (5). This may be due to our use of larger amounts of drug. The mean duration of disease before treatment in this series was 15 days. Dysuria and discharge disappeared on the average in 29 and 82 hours, respectively. In all cases smears were negative for gonococci by the day after treatment. These patients were followed for an average of 28 days during which time there were no relapses.

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THE BENEFICIAL EFFECT OF CHLOROMYCETIN IN BRUCELLOSIS^{1, 2}

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Newer antibiotic agents have greatly extended the range of specific therapy in the group of infectious diseases with increasing rapidity. Much of this effort is directed against gram-negative bacilli in which group the Brucellae command a prominent position. Up to the present time the use of therapeutic agents in brucellosis has resulted in a certain measure of success, but with limitations, particularly from the point of view of failure to prevent recurrences.

One of these new antibiotics is chloramphenicol (Chloromycetin) a streptomycetes, first isolated by Burkholder,⁷ crystallized by Ehrlich and his associates (1) and demonstrated to possess potent antimicrobial effect against many gram-negative organisms including all members of the Brucella group (2). The pharmacological and toxicological characteristics of this new agent are now a matter of record (2) and the chemical structure of the antibiotic agent is known (3). In experimental and clinical infections the synthetic form of Chloromycetin has been found comparable to the naturally produced product in degree of effectiveness (4).

Chloromycetin is well absorbed from the gastrointestinal tract. Serum levels of the drug after oral administration have been found comparable to those obtained by parenteral administration.

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There are no reports up to the present time of toxic manifestations resulting from the oral administration of Chloromycetin. The prolonged intramuscular administration in dogs resulted in a moderately severe anemia without significant changes in the white blood cells and without disturbance in hepatic or renal functions (2).

The initial studies of the antibiotic spectrum of Chloromycetin *in vitro* and *in vivo* in animals indicates a wide range of usefulness, particularly in rickettsial and gram-negative infections (2, 5). Among the gram-negative organisms showing a considerable degree of sensitivity to this antibiotic are *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. It is interesting to point out that up to the present time Chloromycetin has not failed to exert great beneficial effect in those clinical diseases whose organism has been found sensitive to the agent when tested in the laboratory.

In Maryland the opportunity to treat brucellosis afforded itself because of a rather prevalent incidence of the *B. abortus* and *B. suis* types of infection. We have observed nine patients with active infection during the period from July, 1948, to February, 1949. Six of these patients were experiencing an initial attack, whereas three patients showed a relapse of fever two, three and five months after the primary illness treated with a combined course of streptomycin and sulfadiazine. The present study deals with the results obtained following Chloromycetin administration in these nine patients. Observations on non-treated cases (controls) were not made because of the insufficient number of available patients.

METHODS AND PROCEDURE

Chloromycetin was administered orally, the antibiotic having been furnished by Parke, Davis and Company in the form of 0.25 gm. tablets and capsules. The tablets were swallowed whole and in spite of the bitter taste no difficulty was experienced with oral administration. Chlo-

romycetin now supplied in gelatin capsules is taken orally without unpleasantness.

The dosage regimen adopted for this study was empirical and based, in general, on prior experience in scrub typhus and typhoid fever (6, 7). The large initial dose in each instance was approximately 50 mg. per kilo. After the initial dose, Chloromycetin was given at three hour intervals in 0.25 gm. amounts until the temperature was normal and for a minimum of five days thereafter.

Blood levels for Chloromycetin were determined throughout the period of treatment. A modification of the method of Joslyn and Galbraith (8) which employs inhibition of growth of *Shigella sonnei* was used for these micro-biological assays. The sensitivity of the brucella was determined by inoculating a standard quantity of the bacterial suspension into serial dilutions of Chloromycetin in tryptocase broth. After approximately three days' incubation, the sensitivity was determined turbidimetrically by the 50 per cent end-point technique as applied to fluid culture.

Confirmation of the clinical diagnosis of brucellosis was sought in each instance through the following procedures: (1) Blood was taken from the patient for culture before instituting treatment. Venous blood was inoculated directly into tryptocase broth which was then incubated at a temperature of 37° C. in an atmosphere of 10 per cent carbon dioxide. Subcultures were made on tryptocase agar utilizing the same conditions. Blood cultures were repeated on each patient at least three times during the period of hospitalization and one month following discharge from the hospital when the first follow-up studies were performed. (2) Agglutination tests, utilizing *B. abortus* as antigen, were performed by the usual clinical laboratory techniques.

Hemoglobin levels, red and white blood cell counts and differential leukocyte counts were performed in the usual manner.

RESULTS

a. Confirmation of diagnosis: In seven of the nine patients constituting this series of treated pa-

tients, four showed a positive blood culture for *B. abortus*, two for *B. suis* and one for *B. melitensis* prior to the administration of Chloromycetin. All of the post-treatment blood cultures remained sterile. In all nine patients a significantly high rising titer for brucella agglutinins was demonstrated (Table I).

b. Blood levels of Chloromycetin and sensitivity tests: The levels of Chloromycetin in the blood were found to be comparable to those obtained and reported elsewhere (9). For 24 hours following the initial dose of the antibiotic, the level ranged in seven patients tested from 10 to 50 gamma per cc. During the ensuing therapeutic days when the patients were on a comparable dose of .25 gm. every three hours the average level was 15 gamma per cc. up to the time of cessation of therapy. The sensitivity of the infecting organisms tested on four patients ranged from .78 to 2.4 gamma per cc. (Table I). These concentrations compare favorably with the figures reported by Smith *et al.* (2) who had previously demonstrated inhibition of Brucellae to Chloromycetin in the following concentrations: *B. abortus*, 2.0 gamma per cc.; *B. suis* and *B. melitensis*, 0.5 gamma per cc.

The course of undulant fever in patients treated with Chloromycetin

The mean duration of fever prior to treatment in the nine treated patients was 30 days. Two of these patients were more seriously ill than the others. Within 36 hours after the start of specific treatment they were resting more comfortably, spent a more restful night for the first time since

TABLE I
Tabulation of results on the use of Chloromycetin in brucellosis

Case	Age	Sex	Day of illness therapy beg.	Duration fever aft. therapy	Last febrile day of illness	Size of spleen	Chloromycetin		Agglutination		Blood culture	Sensitivity	Maximum blood level
							total days	total grams	Acute	Conv.			
1	27	M	53rd	2.0	55	++	6	13.75	1280	2560	Abortus	2.4	50
2	31	F	12th	2.0	14	+	6	11.25	80	1280	—	—	18
3	32	M	25th	2.5	27	+++	9	19.25	640	640	Suis	—	12
4	28	M	19th	2.0	21	++	6	15.50	320	1280	Suis	1.2	10
5	29	M	23rd	3.0	26	0	8	19.0	640	640	Abortus	.78	33.6
6	38	M	57th	4.5	61	+	12	24.75	1280	1280	Abortus	1.2	24.0
7	24	M	32nd	2.0	34	+	10	22.0	1280	1280	Abortus	—	16.0
8	31	M	25th	2.0	27	0	80	25.0	640	640	—	—	—
9	26	M	28th	5.5	33	++	10	25.0	640	16,000	Melitensis	—	—
Mean			30.3	2.7	33.0		83	20.25					

the onset of illness and diaphoresis was greatly reduced. In the seven other patients there seemed to be immediate improvement of the body and joint pain and there was decidedly improved taste for food.

The mean in the nine patients for duration of fever after beginning Chloromycetin treatment was 2.7 days. The temperature in all remained normal thereafter. Seven of the nine patients had enlarged spleens felt from one to three fingers below the costal margin. In five patients the spleen was observed to become non-palpable during the course of antibiotic treatment and in the remaining two the spleen disappeared to palpation several days after stopping the drug. The response to treatment in six patients is best demonstrated in figures 1-6 which are accompanied by brief clinical records.

Occurrence of complications

a. Relapses: There was one relapse, the recurrence beginning 31 days following discontinuance of Chloromycetin. Although we were unsuccessful in re-isolating the *B. abortus* from the blood, it is of considerable importance that the re-

administration of Chloromycetin resulted in a prompt clinical response.

b. Complications: In the group constituting this series of nine treated patients there were no complications attributed to brucellosis observed after Chloromycetin treatment. The patient with the melitensis type infection developed an orchitis three months following sulfadiazine and streptomycin treatment. Two days after receiving Chloromycetin the patient noted a definite improvement not only in reduction in size of the testicle but a marked reduction in tenderness.

Illustrative Case Reports

Case No. 1. (Figure 1.) The patient was a 27-year-old Negro who contracted illness from drinking raw milk. He was hospitalized at Fort Meade on the 29th day of disease. His early complaints were primarily those of fever, profuse sweating, headache, anorexia, general weakness, and malaise. After admission the evening temperature reached 105° F. and ranged from 102 to 105° prior to the administration of specific therapy. His nights were restless because of the pyrexia, sweating and general aching. There were no signs of a localized infection. On the 32nd day of illness culture of the blood was positive for *B. abortus*. The organism isolated was sensi-

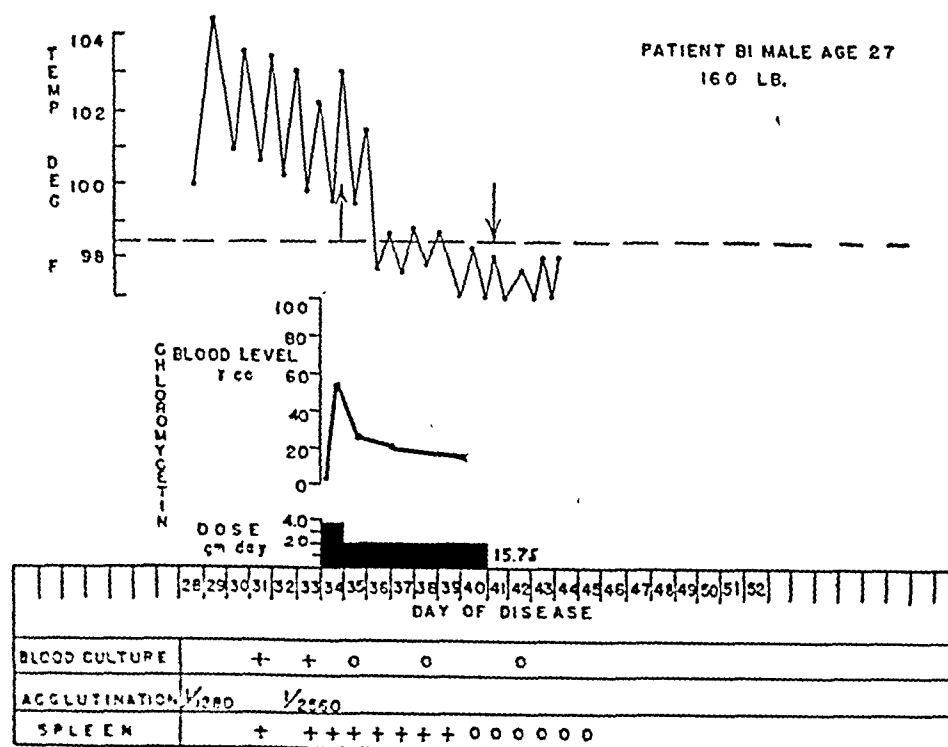


FIG. 1. (CASE NO. 1.) CHART OF RESULTS IN A PATIENT (MALE, AGE 27, 160 LBS.) WITH BRUCELLOSIS OF ABORTUS TYPE—TREATMENT WITH CHLOROMYCETIN

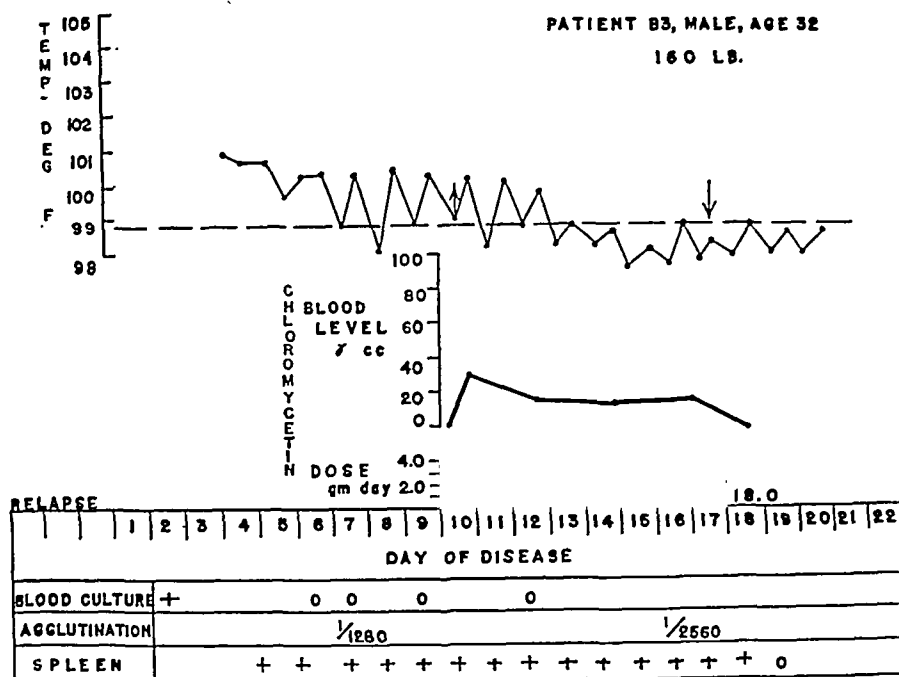


FIG. 2. (CASE No. 3.) THE COURSE OF ILLNESS IN A PATIENT (MALE, AGE 32, 160 LBS.) WITH BRUCELLOSIS OF *SUIS* TYPE—TREATMENT WITH CHLOROMYCETIN

tive to Chloromycetin in the concentration of 2.4 gamma per cc. At this time the serum agglutination for *B. abortus* was 1/2560. The spleen was readily palpable and there were scattered rales throughout both lungs. Chloromycetin was given orally with an initial dose of 3.0 gms. and a subsequent dosage of .25 gm. every three hours. The drug was well tolerated. On the first night of drug treatment the patient was more comfortable and diaphoresis was greatly reduced. On the following day the appetite was decidedly improved and the bedside appearance was brighter. The evening temperature reached normal 48 hours after institution of treatment and remained normal thereafter. The spleen could not be felt after five days of therapy. A total of 15.75 gms. of Chloromycetin was administered, the course extending until five days of normal temperature. Three blood cultures taken during the convalescent period were sterile. The patient was discharged 25 days after the institution of Chloromycetin and now remains free of symptoms.

Case No. 3. (Figure 2.) A 32-year-old white man first became ill in June, 1948, with evening fever, anorexia, general malaise, weakness and chilly sensations. For one year the patient had been employed in a slaughter house participating in the slaughtering of cattle, hogs and in the packing of meat. For the initial attack he received combined streptomycin and sulfadiazine. At this time the spleen was enlarged, firm and smooth to palpation and descended two finger breadths below the costal margin. The agglutinins for *B. abortus* rose from 1/1280 to 1/10240. The temperature became normal after seven days of this combined treatment but the spleen did not

appreciably change in size. Two months later the patient noticed a recurrence of symptoms, the evening temperature reached 104° F. and the spleen was readily palpable. The serum agglutination titer was 1/1280 and the blood culture was positive for *B. suis*. Chloromycetin was administered with an initial dose of 50 mg. per kilo. beginning on the 10th day of relapse. The drug was then administered orally in .25 gm. amounts every three hours until five days of normal temperature. The evening temperature reached normal 2.5 days following the institution of treatment, at which time the patient was asymptomatic. In six weeks the patient had no complaints, the blood cultures were sterile and the spleen was not palpable. The patient is now eight months convalescent and apparently well.

Case No. 4. (Figure 3.) A 28-year-old male contracted *B. suis* infection from handling meat in a city packing establishment. The primary attack began in June, 1948, at which time the blood culture revealed *B. suis*. The spleen was palpable on this admission. The evening temperature averaged 101°, and the patient complained primarily of general muscular aching along with sweating and anorexia. After three weeks of temperature a combined course of streptomycin and sulfadiazine was administered. The temperature reached normal in seven days, and the patient was generally improved. Brucellin was employed during the convalescent period which was accompanied by body pain and fever soon after administration. A persistent daily fever reappeared less than two months after the first therapeutic course. The blood revealed *B. suis* and the serum agglutinating titer

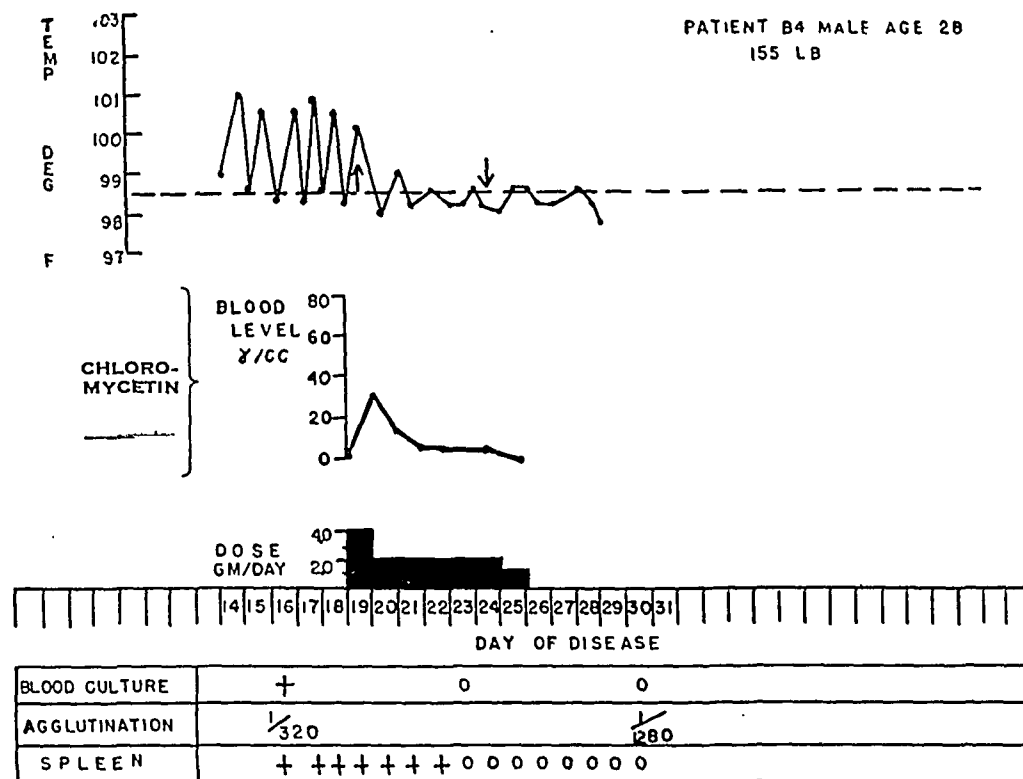


FIG. 3. (CASE No. 4.) CHART OF RESULTS IN A PATIENT (MALE, AGE 28, 155 LBS.) WITH BRUCELLOSIS, SUIS TYPE—TREATMENT WITH CHLOROMYCETIN
No recurrence after eight months.

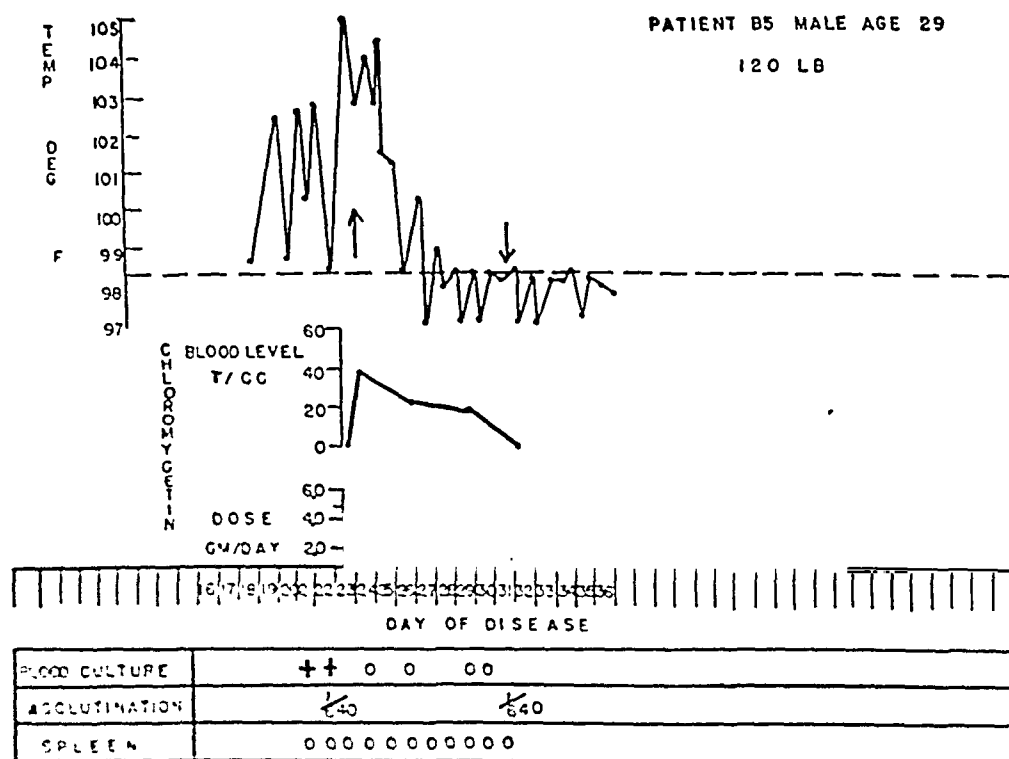


FIG. 4. (CASE No. 5.) THE RESPONSE TO CHLOROMYCETIN IN A PATIENT (MALE, AGE 29, 120 LBS.) SEVERELY ILL WITH BRUCELLOSIS (ABORTUS TYPE)
No recurrence after six months.

Case No. 5. (Figure 4.) A 29-year-old white man contracted undulant fever from drinking raw milk. The acute illness was characterized by upper abdominal pain, nocturnal fever, anorexia and general malaise. Blood taken on the 22nd and 23rd days of illness revealed *B. abortus* found sensitive to Chloromycetin in the dilution of .78 gamma per cc. The serum agglutinating titer was 1/640. Before institution of treatment the evening temperature ranged from 102 to 104.5° F., the patient appeared quite toxic and had little inclination to eat. Sweating was moderately severe. Three gms. of Chloromycetin were given orally on the 23rd febrile day. The subsequent oral dose was .25 gm. every three hours. The temperature reached normal after three days of anti-

Case No. 6. (Figure 5.) A 38-year-old white man acquired *B. abortus* infection from drinking unpasteurized milk in a rural area. The early illness was characterized by evening temperature, chilly sensations and severe sweating. There was upper abdominal pain with general malaise and bothersome joint pains. Anorexia was marked. On admission to the hospital on about the 50th day of illness a rather toxic individual was observed. The temperature reached 104° F. Examination revealed a few scattered rales in the lungs and an easily palpable spleen. The agglutination for *B. abortus* was 1/1280. Two blood cultures taken during his pretreatment study period were positive for *B. abortus* which was found sensitive to Chloromycetin in the dilution of 1.2 gamma per cc. Three gms. of Chloromycetin were administered on the 57th day of illness with subsequent doses of .25 gm. every three hours until five days of normal temperature. There was no fever after 4.5 days of treatment.

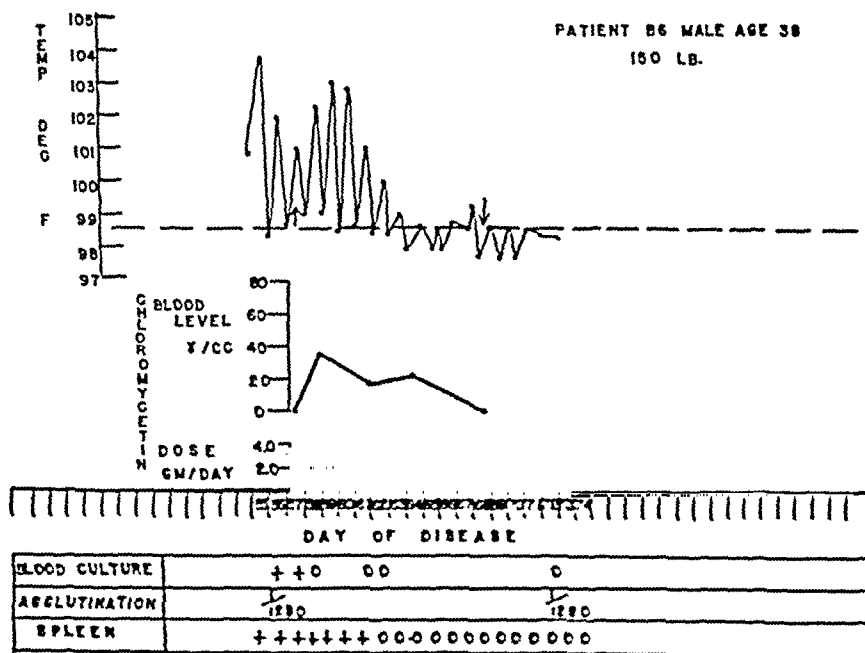


FIG. 5. (CASE NO. 6.) COURSE OF ILLNESS IN A PATIENT (MALE, AGE 24, 150 LBS.) WITH ANGIOTIC TYPE OF BINGHAMOSIS—TREATMENT WITH CHLOROMYCETIN

No recurrence after seven months.

sial diseases (6, 11-13) is further evidence to suggest that this antibiotic might be expected to exert beneficial effect in brucellosis.

Spink and his collaborators (14) utilized the fertilized egg to demonstrate that a combination of streptomycin and sulfadiazine was sufficient to eradicate *Brucellae* from the tissues of infected embryos whereas they found that either drug used alone was incapable of achieving this effect. The clinical use of these combined agents in patients with brucellosis yielded more satisfactory results than had any prior treatment. In a series of treated cases these investigators (15) state that in the febrile patient normal temperatures were not sustained until the seventh to 10th days of treatment. Relapses with this form of treatment have been observed by others, and in our series of nine patients three had received treatment at an earlier date with the streptomycin and sulfadiazine regime, and were in active relapse with positive blood cultures when placed under therapy with the new antibiotic.

Detailed accounts of the successful treatment of brucellosis with aureomycin have been reported (16-18). In 24 patients with the more severe *B. melitensis* infection, Spink and his associates observed prompt reduction in toxemia and return of temperature to normal in 72 hours. Three relapses were encountered in this series up to the time of writing. In five patients with brucellosis, two proven to be abortus in type by blood cultures, we observed similar clinical improvement after aureomycin treatment (19). The temperature returned to normal in three days.

With Chloromycetin treatment in nine cases of active brucellosis we have observed abatement of symptoms within a short period and return of temperature to normal in three days after instituting treatment (Table I). One relapse occurred 30 days after discontinuing Chloromycetin. The response to retreatment was prompt. Only one patient with the more virulent *melitensis* type of brucellosis has been treated with this antibiotic. This patient responded favorably with return of temperature to normal in five days. It thus appears that both Chloromycetin and aureomycin exert similar favorable effect in amelioration of the symptoms of this disease, and it is not possible to affirm from the still limited accumulated evidence

which antibiotic is more effective. Long term observation of a large series of patients, varied treatment schedules and observations for possible toxic effects will need study before proper evaluation of these agents can be determined in a disease so variable in its clinical manifestations. Undoubtedly, until the optimum course of treatment is determined, additional relapses will occur at which time it will be highly instructive to observe the clinical effects after reinstituting Chloromycetin and to compare the drug sensitivity of the organism causing the relapse with the one isolated during the initial attack. It is probable that longer courses of antibiotic treatment may be necessary or perhaps periodic courses of antibiotic until a sufficient degree of immunity has developed.

CONCLUSIONS

Chloromycetin has been found to exert specific beneficial effect in nine patients with active brucella infection.

Further clinical testing will be necessary to determine the optimum therapeutic regimen.

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THE EFFECT OF AUREOMYCIN AGAINST *BACTERIUM TULARENSE*¹

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The wide antibiotic spectrum of aureomycin prompted exploration for possible activity against *B. tularensis*. Its evidently low toxicity and its absorption in active form when administered orally would be decided advantages should it prove to be effective in tularemia.

EXPERIMENTAL STUDIES

The test organism. Studies were undertaken using a virulent strain of *B. tularensis* recently recovered from a patient who died of tularemic meningitis (1). The organism was agglutinated by known tularemic serum to full titer. It failed to grow on blood agar but grew well on solid medium containing 8% fresh rabbit blood, 1% dextrose, and 0.1% l-cystine in beef heart infusion agar base, prepared according to the method of Francis (2). Early cultures were preserved at -20° C. or 4° C. Fresh 24 to 48 hour transplants on blood dextrose cystine agar were used in all experiments, representing the second to seventh transfers from the original culture. The purity of each culture used was checked microscopically and on blood agar plates.

1. Inhibition of *B. tularensis* by aureomycin in culture medium

Method: Fresh medium was prepared by adding 8% rabbit blood to dextrose cystine beef heart infusion agar at 60° C. For comparison with control medium, aureomycin-HCl² from 20 mg. vials was dissolved in distilled water and introduced into flasks of this medium to yield final concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 micrograms per ml. Exposure of the drug to 60° C. was momentary. The medium was quickly tubed, slanted, and hardened in the icebox. The elapsed time between dissolving the aureomycin and inoculation of the slants was five to six hours. The final pH of the medium was 6.8, and was not changed detectably by addition of aureomycin-HCl in concentration of 64 micrograms per ml.

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² The aureomycin used in these studies was supplied through the courtesy of Lederle Laboratories Division, American Cyanamid Co.

The effectiveness of aureomycin in this medium against *B. tularensis* and *Staph. aureus* was tested. The staphylococcus used was inhibited by 1.5 but not by 0.75 micrograms of aureomycin per ml. in tryptose phosphate broth of pH 7.2 at 24 hours incubation. Turbid suspensions in broth were made from 26 hour blood dextrose cystine agar cultures of each organism. Slants containing 0 to 8 micrograms aureomycin per ml. were inoculated with 1 loopful of *Staph. aureus* or 3 loopfuls of *B. tularensis* and incubated at 37° C.

Results: Growth of *B. tularensis* did not become apparent on the control medium until 24 hours, at which time 0.5 microgram of aureomycin per ml. caused complete inhibition, while *Staph. aureus* required 1 microgram per ml. for inhibition (Table I). These early results are difficult to interpret because *B. tularensis* grows more slowly. After 36 hours the concentrations of aureomycin

TABLE I
INHIBITION OF *B. TULARENSE* AND *STAPH. AUREUS* BY
AUREOMYCIN IN BLOOD DEXTROSE CYSTINE AGAR SLANTS

ORGANISM	HOURS INCUBATION AT 37° C.					
	12	24	36	48	67	96
	CONC. OF AUREOMYCIN CAUSING COMPLETE INHIBITION					
<i>STAPH. AUREUS</i> *	0.5	1.0	2.0	2.0	4.0	>8.0
<i>B. TULARENSE</i>	?	0.5	2.0	2.0	4.0	>8.0

* THIS STRAIN IS INHIBITED BY AUREOMYCIN 1.5 MICROGRAMS PER ML. IN TRYPTOSE PHOSPHATE BROTH AT 24 HOURS INCUBATION

required to inhibit the two organisms were comparable, approximately doubling each 24 hours until 96 hours when growth of both was observed with 8 micrograms per ml. Earlier experiments showed that neither organism grew on slants containing 16 or more micrograms per ml. after incubation for one week.

Aureomycin in concentrations of 8 micrograms or less per ml. thus inhibited but did not kill *B. tularensis* and *Staph. aureus* inoculated on blood dextrose cystine agar slants. The progressive

daily increase in the concentration required for inhibition is best explained by deterioration of the drug on incubation at 37° C., as shown by Paine *et al.* (3) and Bliss and Chandler (4). The strain of *B. tularensis* tested was inhibited by concentrations comparable to those required for inhibition of a sensitive strain of *Staph. aureus*.

2. Effect of incubating *B. tularensis* with aureomycin in broth

Method: A turbid suspension of *B. tularensis* with a density of 4 on the McFarland scale (5) was prepared in tryptose phosphate broth from a 41 hour blood dextrose cystine agar culture. Aliquots of this suspension were added to equal volumes of broth, of aureomycin-HCl 100 micrograms per ml. in distilled water, and aureomycin 1000 micrograms per ml. in distilled water yielding final aureomycin concentrations of 0, 50, and 500 micrograms per ml. The initial pH of the broth was 7.2 and was not changed detectably by addition of aureomycin 50 micrograms per ml. It was reduced only to 7.05 by addition of 500 micrograms per ml. This is well within the pH range for maximal growth of *B. tularensis*. The suspensions were incubated at 37° C. for four hours, after which tenfold serial dilutions in broth were made.

Results: Inocula of 0.1 ml. of dilutions 10^{-1} to 10^{-10} were injected intraperitoneally into groups of four or five mice (Table II). All mice receiving dilutions 10^{-1} to 10^{-6} inclusive died. The control suspension incubated with broth had an LD₅₀ of 10^{-9} by the method of Reed and Muench (6). When incubated with 50 micrograms of aureomycin per ml. the LD₅₀ was $10^{-8.3}$ and with 500 micrograms per ml. it was $10^{-7.2}$.

Slants of blood dextrose cystine agar were also inoculated with three loopfuls of 10^{-1} and 10^{-3} dilu-

tions prepared from the three suspensions after incubation as described above. When incubated three days at 37° C. the slants from both dilutions of the control broth suspension and from the 10^{-1} dilution of the suspension containing aureomycin 50 micrograms per ml. showed a heavy confluent growth of *B. tularensis*. The 10^{-3} dilution of the suspension containing aureomycin 50 micrograms per ml. and the 10^{-1} dilution of the suspension containing 500 micrograms per ml. yielded innumerable separate colonies. The 10^{-3} dilution of the latter gave only 10-12 small colonies. Although after one week 65 colonies were visible, growth was still strikingly reduced as compared with cultures from the other suspensions.

It is evident that incubating a suspension of *B. tularensis* for four hours at 37° C. in the presence of aureomycin 500 micrograms per ml. killed an appreciable number of organisms. Questionable reduction of viable bacteria occurred in the presence of 50 micrograms per ml.

3. Subcutaneous aureomycin therapy of experimental tularemia in mice

Method: In therapeutic experiments standard suspensions of *B. tularensis* were freshly prepared by emulsifying growth from a 24 to 48 hour blood dextrose cystine agar culture in sterile saline and adjusting to a density of two on the McFarland scale (5). All suspensions were cultured on blood agar for purity and yielded no growth. Tenfold serial dilutions in broth were made. Young adult white male mice were inoculated intraperitoneally with 0.1 ml. of various serial dilutions. Control mice and those to be treated were inoculated with the same dilution. Simultaneously the LD₅₀ of the suspension was determined by titration in additional groups of mice. Aureomycin-HCl dissolved in sterile distilled water was administered subcutaneously to the mice designated for treatment. Mouse tularemia is apparently not contagious to cage mates except perhaps by cannibalism, which was prevented so far as possible by prompt removal of dead animals.

Procedure and Results: A pilot experiment was performed to determine the LD₅₀ for mice of a standard suspension of *B. tularensis*. Two of the higher serial dilutions were arbitrarily chosen for treatment (Table III). Inocula of 0.1 ml. of dilutions 10^{-4} to 10^{-9} inclusive were injected intraperitoneally into groups of four mice. Additional groups of four mice received the 10^{-7} and 10^{-8} dilutions. Treatment of the two latter groups with subcutaneous injections of aureomycin-HCl 0.5

TABLE II

EFFECT OF INCUBATING A BROTH SUSPENSION OF *B. TULARENSIS* WITH AUREOMYCIN (4 HOURS AT 37°C) UPON INFECTIVITY FOR MICE

CONCENTRATION OF AUREOMYCIN / ML.	DILUTION INOCULATED (0.1 ML. I-P)					50% MORTALITY TITER
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
0	0/4 ¹	0/1	0/4	2/4	4/4	10^{-9}
50	0/5	0/4	1/4	4/4	4/4	$10^{-8.3}$
500	0/5	3/5	5/5	3/5	5/5	$10^{-7.2}$

¹ALL MICE INOCULATED WITH DILUTIONS 10^{-1} TO 10^{-6} DIED
²NUMBER INOCULATING FLUIDS
³NUMBER INOCULATED

TABLE III

PROTECTIVE EFFECT OF AUREOMYCIN ADMINISTERED SUBCUTANEOUSLY TO MICE INFECTED WITH SMALL INTRAPERITONEAL INOCULA OF *B. TULARENSE*

TREATMENT	DILUTION INOCULATED (0.1 ML I-P)				50% MORTALITY TITER
	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
NONE	0/4 ¹	0/4	0/4	3/4	10 ^{-8.7}
15 MG/DAY S-C, BEGUN IMMEDIATELY, 5 DAYS	—	4/4 10+ MLD	4/4 1+ MLD	—	—

¹NUMBER OF MICE SURVIVING 21 DAYS
NUMBER INOCULATED

mg. was begun immediately and continued three times daily at approximately eight hour intervals for five days, a total of 16 injections. (Each dose consisted of 0.25 ml. of a solution containing 2 mg. of aureomycin-HCl per ml. in distilled water.) All untreated mice inoculated with dilutions 10⁻⁴ to 10⁻⁸ inclusive and one receiving 10⁻⁹ died. The LD₅₀ of the suspension was 10^{-8.7}. All treated mice inoculated with the 10⁻⁷ and 10⁻⁸ dilutions survived 27 days after inoculation. It was evident that the inocula were small, about 10 and 1 MLD respectively, but 100% survival of the treated animals was noteworthy.

To investigate the possibility that *B. tularensis* was harbored in the tissues of the surviving treated animals, two mice were killed on the 27th day after inoculation with the 10⁻⁷ dilution. The spleen of one was enlarged to at least three times normal size, dark red, and showed definite pale focal areas on section. The spleen of the other was enlarged to perhaps twice normal size but showed only questionable foci. No other gross changes were noted. Spleen impression smears stained by Gram's method were negative for *B. tularensis*. The spleens were ground with sterile sand and emulsified in broth. Cultures yielded no growth on blood agar and blood dextrose cystine agar. Five mice were inoculated intraperitoneally with 0.1 ml. One died on the third day and one on the 12th, apparently neither of tularemia as shown by absence of typical autopsy findings and by negative spleen impression smears. The other three mice survived for 21 days. Accordingly, no evidence was obtained in this experiment to suggest that viable *B. tularensis* persisted in the

spleens of mice which survived as a result of aureomycin treatment.

To investigate the possible development of immunity in treated animals, the remaining six mice which had survived inoculation with the 10⁻⁷ and 10⁻⁸ dilutions were reinoculated intraperitoneally on the 27th day with 10⁻⁶ dilution of a suspension of *B. tularensis* used in a later experiment (see Table V). The inoculum represented 10+ MLD. All mice died on the third or fourth day after reinoculation. Autopsies on three showed typical changes of tularemia. Spleen impression smears were positive for *B. tularensis*, and the organism was recovered in pure culture from the spleens and heart bloods. There is thus no evidence that mice surviving inoculation with *B. tularensis* for 27 days as a result of aureomycin treatment had any appreciable immunity on reinoculation.

A second experiment was undertaken to determine the effect of smaller subcutaneous doses of aureomycin (Table IV). The LD₅₀ of the suspension was 10^{-8.5}. Groups of mice were inoculated with 10⁻⁶ dilution, and treatment was begun two hours later. All of 10 untreated mice died on the third or fourth day. Animals treated with 0.5, 0.25, 0.125, and 0.065 mg. of aureomycin subcutaneously three times daily at about eight hour intervals for a total of 16 doses (i.e., 1.5 to 0.19 mg./day, or approximately 75 to 9.4 mg./kg./day) remained well during five days therapy but all except three of 37 animals died after treatment was discontinued. That the deaths were due to tularemia was confirmed by positive autopsy findings and spleen impression smears. Mice receiving the largest dose of aureomycin (1.5 mg./day) survived longest. It did not seem feasible to

TABLE IV

EFFECT OF SUBCUTANEOUS AUREOMYCIN THERAPY BEGUN 2 HOURS AFTER INTRAPERITONEAL INOCULATION OF MICE WITH *B. TULARENSE*

DOSE, S-C MG./DOSE	NO. MICE TREATED	DEATHS AFTER TREATMENT				
		DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
0	10	1	1	1	1	1
0.5	8	1	1	1	1	1
0.25	10	1	1	1	1	1
0.125	10	1	1	1	1	1
0.065	9	1	1	1	1	1

LD₅₀ OF SUSPENSION 10^{-8.5}
INOCULUM 0.1 ML. OF 10⁻⁶ = 10+ MLD

use larger subcutaneous doses since edema, induration, and local necrosis were not infrequently noted following repeated injections of 0.5 mg. of aureomycin-HCl in 0.25 or 0.2 ml. of distilled water (2 to 2.5 mg./ml.). Local reactions were inconspicuous with concentrations of 1 mg./ml. or less.

The effect of duration and time of starting subcutaneous aureomycin treatment were next investigated (Table V). The LD_{50} of the suspension was $10^{-8.2}$. All of 20 control mice receiving the 10^{-6} dilution died on the third or fourth day. Groups of 10 mice which received the same inoculum were given 0.5 mg. of aureomycin subcutaneously at about eight hour intervals (1.5 mg./day or approximately 75 mg./kg./day) for 16 injections over a period of five days. One group received the first injection immediately before inoculation. A second group was started on therapy seven hours after inoculation. Two additional groups were given the same dosage of aureomycin started at the same times, but were continued on treatment for eight days or a total of 25 injections. All except one of the 40 treated mice remained well during therapy, but deaths began to occur on the fourth to sixth day after cessation of treatment and relatively few animals survived. Half of the group in which therapy was started just before inoculation and continued for eight days lived.

Two additional groups of 10 mice each, not shown in Table V, were included in this experiment and received the same inoculum at the same time. Treatment by subcutaneous injection was deferred until 28 hours after inoculation and continued for five and eight days respectively. These animals were likewise protected during therapy but deaths began to occur on the fifth day after cessation of treatment. All except one in

the group treated five days and two in the group treated eight days succumbed.

The best result (50% survival) was thus obtained when subcutaneous therapy was begun immediately before inoculation and continued for the longest period, *i.e.*, eight days. Whether more complete protection could be obtained with larger doses administered for a longer period, or with repeated short courses of treatment, is not known.

4. Oral aureomycin therapy of experimental tularemia in mice

Methods: Powdered drug from capsules was mixed with dry finely-ground Rockland mouse diet to yield concentrations of 0.5%, 0.25%, 0.125%, and 0.065%. Food was withheld for six hours and mice were then given the drug-containing diet *ad libitum*. By weighing the food taken it was found that 10 mice on the diet containing aureomycin 0.125%, inoculated intraperitoneally with *B. tularensis* on the second day, consumed an average of 3.68 gm. per mouse per day over an eight day period. If the average consumption be assumed to approximate 3.5 gm. per mouse per day, the amount of aureomycin taken in these diets may be estimated roughly as follows:

% of aureomycin in diet	Estimated amount of aureomycin consumed per mouse per day
%	mg.
0.5	17.5
0.25	8.8
0.125	4.4
0.065	2.2

In therapeutic experiments the drug-containing diet was begun, after a six hour fast, on the day prior to intraperitoneal inoculation with *B. tularensis*.

Procedure and Results: Groups of 10 mice were started on diets containing aureomycin 0.5%, 0.25%, 0.125%, and 0.065% while 20 control mice were given the basic diet without aureomycin. After 22 hours all mice were inoculated with a 10^{-6} dilution of a suspension of *B. tularensis* (Table VI). The LD_{50} of the suspension was $10^{-8.2}$. The treated animals were fed the aureomycin-containing diets for 14 days. All of the control mice died on the third or fourth day after inoculation. None of the animals receiving drug in the diet died during 14 days of therapy, but deaths began to occur on the third to fifth days after the basic ration was resumed. Nevertheless, significant numbers remained alive; a survival rate of 60% was observed in the mice fed diet containing 0.5% of aureomycin, with somewhat fewer survivals in the other groups. These results compared favorably with those observed after subcutaneous treatment.

TABLE V

EFFECT OF DURATION AND TIME OF STARTING AUREOMYCIN THERAPY (1.5 MG/D S-C) IN MICE INOCULATED INTRAPERITONEALLY WITH *B. TULARENSE*

TREATMENT	NO. MICE	DEATHS BY DAYS AFTER INOCULATION					
		DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
NONE	20	10	10				
0.5% (S-C)	10	0	0	0	0	0	0
0.25% (S-C)	10	0	0	0	0	0	0
0.125% (S-C)	10	0	0	0	0	0	0
0.065% (S-C)	10	0	0	0	0	0	0

NOTE: 1.5 MG/D S-C = 1.5 MG. AUREOMYCIN-HCL IN 0.25 ML. DISTILLED WATER

TABLE VI
EFFECT OF AUREOMYCIN IN DIET OF MICE INOCULATED
INTRAPERITONEALLY WITH *B. TULARENSE*

% AUREOMYCIN IN DIET	NO MICE INOC.	DEATHS BY DAYS AFTER INOCULATION						
		DAYS						
		5	10	15	20	25	30	
0	20	DEATHS 14 6						
0.065	10				1 1 2	1		
0.125	10				1 3	1	1	1
0.25	10				1 1 2		1	1
0.5	10				1	2	1	

LD₅₀ OF SUSPENSION, 10^{-8.2}
INOCULUM 0.1 ML OF 10⁻⁶ I-P (10⁵ MLD)
DIET STARTED 22 HOURS BEFORE INOCULATION

In studies on aureomycin therapy of relapsing fever, Heilman (7) found that the oral dosage required was in the order of five times the subcutaneous dose. If the same ratio pertains in tularemia, the diet containing 0.25% aureomycin might be estimated to be about equivalent to subcutaneous dosage of 1.5 mg. daily. In our experiments the results obtained with this diet fed for 14 days approximated those with subcutaneous injections of 1.5 mg. daily for eight days beginning immediately before inoculation (cf. Tables V and VI).

A second feeding experiment was undertaken to investigate the effectiveness of aureomycin in suppressing larger inocula (Table VII). Six groups of 10 mice were started on a diet containing 0.065% aureomycin. After 22 hours these were inoculated with the 10⁻² to 10⁻⁷ dilutions of a suspension of *B. tularense*. Control groups of six mice fed on basic ration without aureomycin were simultaneously inoculated with the 10⁻² to 10⁻¹⁰ dilutions. The treated animals received the

TABLE VII
SUPPRESSIVE EFFECT OF AUREOMYCIN 0.065% IN DIET OF
MICE INFECTED WITH VARYING INOCULA OF *B. TULARENSE*

DILN POC	NO MICE	DIET	DEATHS BY DAYS AFTER INOCULATION						
			DAYS						
			5	10	15	20	25	30	
10 ⁻²	10	NORMAL RATION	3 1						100000 MLD
10 ⁻³	10		2 4						100000 MLD
10 ⁻⁴	10		1 5						10000 MLD
10 ⁻⁵	10		4 2						10000 MLD
10 ⁻⁶	10		1 6						1000 MLD
10 ⁻⁷	10		1 6						100 MLD
10 ⁻⁸	10	AUREO DIET	1 1	1 1	2 2	1 1			
10 ⁻⁹	10		1 1	1 1	1 1	1 1			
10 ⁻¹⁰	10		1 1	1 1	1 1	1 1			
10 ⁻¹¹	10		1 1	1 1	1 1	1 1			
10 ⁻¹²	10		1 1	1 1	1 1	1 1			
10 ⁻¹³	10		1 1	1 1	1 1	1 1			

LD₅₀ OF SUSPENSION, 10^{-8.2}
INOCULATED MICE RECEIVING 10⁵ MLD
DIET STARTED 22 HOURS BEFORE INOCULATION

drug-containing diet until eight days after inoculation, when it was replaced by basic ration. The LD₅₀ of the suspension was 10^{-8.2}. All of the control mice inoculated with the 10⁻² to 10⁻⁸ dilutions died on or before the fifth day. In the group of treated mice which received the 10⁻² dilution, representing about 1,000,000 MLD, five deaths occurred during the eight day treatment period, but only one of 50 mice in the other groups died during therapy. Partial suppression of tularemia was thus demonstrated by aureomycin against 1,000,000 MLD, and almost complete suppression was observed with inocula ranging from 10 to 100,000 MLD. However, after the drug-containing diet was withdrawn deaths began to occur and most of the animals succumbed by the 13th day after inoculation. The greatest percentage of survivals was noted in the group given the smallest inoculum, i.e., the 10⁻⁷ dilution, which contained about 10 MLD.

The size of inoculum appeared to be a decisive factor in determining ultimate survival of mice infected intraperitoneally with *B. tularense* and given aureomycin therapy, either orally or subcutaneously. With small inocula ranging from one to 10 MLD a very significant number of animals remained well. Although the drug exerted a striking suppressive effect against inocula up to 100,000 MLD, few mice which received inocula approaching or exceeding 100 MLD survived.

DISCUSSION

The first successful chemotherapy of experimental tularemia was reported by Heilman (8). He administered streptomycin subcutaneously to mice in divided doses totalling 1 mg. daily. Four injections of 0.15 mg. in saline and one of 0.4 mg. in beeswax and sesame oil were given in each 24 hours, and continued for 10 days beginning seven or eight hours after intraperitoneal injection of *B. tularense*. This regimen protected mice against inocula estimated at 10⁴ to 100⁴ MLD. When the total daily dose of streptomycin was reduced to 0.5 mg., only five of 12 mice were protected against 100⁴ MLD. The strain of *B. tularense* used was reported to be completely inhibited by 0.15 microgram of streptomycin per ml. *in vitro*. Because of differences in dosage form and schedule it is not possible to make a direct comparison between the results with aureomycin

reported herein and those obtained by Heilman using streptomycin.

Comparative studies on the activity of aureomycin, streptomycin, and chloromycetin in experimental tularemia have recently been reported by Woodward *et al.* (9). Using larger doses of aureomycin (3 mg./day intramuscularly for four days) they also occasionally obtained 100% survival of mice infected intraperitoneally with small inocula, but with larger inocula death regularly occurred after cessation of aureomycin treatment. They concluded, however, that aureomycin was more effective than streptomycin or chloromycetin in delaying death of mice infected with *B. tularensis*.

Because experimental tularemia in the mouse is an overwhelming septicemic disease with 100% mortality, it is a severe test of a chemotherapeutic agent. It is difficult to apply the results obtained to the human disease, either in general terms or in the more delicate matter of dosage required. When significant activity against *B. tularensis* has been established, both *in vitro* and in mice, carefully controlled clinical trial is the only means of evaluating the usefulness of a new antibiotic in human tularemia.

Woodward *et al.* (9) reported three tularemia patients in whom aureomycin was considered to be effective, and we have also treated three cases with apparently good results (1). Further cautious clinical trial of aureomycin in human tularemia is strongly indicated.

SUMMARY

Aureomycin exerted definite activity against a virulent strain of *B. tularensis*, *in vitro* and *in vivo*. When the drug was incorporated into blood dextrose cystine agar, *B. tularensis* was inhibited by 0.5 microgram per ml. at 24 hours and 2 micrograms per ml. at 48 hours. Its sensitivity was comparable to that of a strain of *Staph. aureus*. In concentrations of 8 micrograms or less per ml., aureomycin was bacteriostatic. However, when a suspension of *B. tularensis* was incubated for four hours in the presence of 500 micrograms per ml., appreciable numbers of organisms were killed as demonstrated by culture and titration in mice.

Furthermore, aureomycin administered subcutaneously or orally demonstrated a striking sup-

pressive effect in mice inoculated intraperitoneally with *B. tularensis*. Almost all animals survived treatment periods ranging from five to 14 days, but most of them died after cessation of therapy. With inocula between 10 and 100 MLD, about 50% survival resulted from subcutaneous therapy with 1.5 mg./day (75 mg./kg./day) given in three divided doses starting immediately before inoculation and continued at about eight hour intervals for eight days. Treatment achieved 100% protection in small groups of mice inoculated with 1 to 10 MLD.

Administration of 0.065% aureomycin in the diet of mice infected intraperitoneally with *B. tularensis* produced partial suppression of 1,000,000 MLD and almost complete suppression of 10 to 100,000 MLD during eight days therapy but most of the animals died after treatment was discontinued. Four of ten treated animals receiving only 10 MLD survived.

A careful trial of aureomycin in therapy of human tularemia is indicated.

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THE CONCENTRATION OF AUREOMYCIN IN URINE AND CEREBROSPINAL, PLEURAL AND ASCITIC FLUIDS AFTER ORAL AND INTRAVENOUS ADMINISTRATION¹

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During our previously reported studies (1) on the absorption of aureomycin in humans after oral and intramuscular administration, we found that aureomycin was sometimes present in the cerebrospinal fluid. We have extended these studies in order to determine the conditions under which aureomycin will be detectable. The present paper includes the cerebrospinal fluid findings in 26 subjects with no inflammation of the central nervous system and in one patient with meningitis and two with encephalitis together with a small number of tests for aureomycin concentration in urine and in pleural and ascitic fluids.

METHOD

Cerebrospinal fluid specimens were obtained simultaneously with specimens of serum on two patients after a single dose of 2 gm. by mouth, on four after a single 1 gm. dose, and on 18 patients who were under treatment with aureomycin² in doses of 250 to 500 mg. every three hours by mouth. Five subjects were given a single intravenous dose of 500 mg. of aureomycin and a single specimen of blood and spinal fluid was taken three or four hours later. One patient with a pleural effusion was given oral aureomycin and simultaneous blood and pleural fluid specimens were obtained. The same procedure was followed in the case of a patient with cirrhosis and ascites.

Aureomycin concentrations were determined by a modification of Dornbush's (2) method, as previously described by us (1).

RESULTS

Distribution into the Cerebrospinal Fluid. As shown in Figure 1, aureomycin was present in the cerebrospinal fluid in only one among 17 specimens obtained within the first 12 hours of aureomycin therapy. Five patients were given a single

intravenous dose of 500 mg. of aureomycin in 25 cc. of arginine or leucine buffer solution. Three to four hours later the serum aureomycin concentration was 2 μ g. in each instance. At the same time 0.06 μ g. was present in the cerebrospinal fluid of one patient and 0.03 μ g. in the fluid of two patients, while detectable amounts of aureomycin were not demonstrated in the cerebrospinal fluids of the two remaining patients.

All of the 16 specimens of cerebrospinal fluid obtained from patients who had received the antibiotic for 23 hours or more contained demonstrable concentrations of aureomycin. The presence of meningitis apparently made no difference in the cerebrospinal fluid concentration attained. Two specimens obtained from patients with encephalitis contained unusually high cerebrospinal fluid concentrations compared with serum concentrations. In general, the higher the serum concentration the higher the concentration in the cerebrospinal fluid. There was no indication that after the first 24 hours there was any further rise in the spinal fluid concentration unless the dose of aureomycin was increased sufficiently to cause a rise in the serum concentration.

Distribution in Pleural and Ascitic Fluids. One patient with a pleural effusion had a concentration of aureomycin in the serum of 0.5 μ g./cc. and 0.125 μ g./cc. in the pleural fluid (Table I). Another patient with cirrhosis and ascites had concentrations of aureomycin in the ascitic fluid of 0.125 and 1.0 μ g. at the same time that serum concentrations were 0.5 and 1 μ g., respectively. A third concentration of 0.03 μ g. was obtained at a time when a simultaneous serum level was not done.

Excretion in the Urine. The amount of aureomycin excreted in the urine after oral and intravenous administration is shown in Table II. One patient was given a single dose of 1 gm. and another 700 mg. by mouth. The concentration of the antibiotic in the urine was 64 to 128 μ g. cc. in

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² Supplied by the Lederle Laboratories Division, American Cyanamid Co.

TABLE I

Distribution of aureomycin in pleural and ascitic fluids

Patient	Diagnosis	Day of treatment*	Concentration of aureomycin $\mu\text{g./cc.}$	
			Serum	Serous cavity fluid
L. C.	Pleural effusion (probably tuberculosis)	2	0.5	0.125
E. Q.	Cirrhosis with ascites	2	0.5	0.125
		3	ND	0.03
		4	1.0	1.0

* With 250 mg. of aureomycin every three hours

both instances and the urinary output during six hours was 1095 and 1060 cc., respectively. The percentage of the dose recovered in the first six hours was 11 and 15.4 per cent, respectively.

Five patients were given a single intravenous dose of 300 mg. One patient (J. M.) had low concentrations in the blood and in the urine and also

a lower urine volume than the two patients described above. In the first six hours he excreted only 4 per cent of the dose administered. The other four patients had higher serum and urine concentrations. One of these excreted 2 per cent in two hours and the other three excreted 5, 2.3, and 3.7 per cent, respectively, in one hour.

The most interesting finding was the relationship between the serum and the urine concentrations, which was remarkably constant considering the crudeness of the biological test employed. The ratio of serum concentration to urine concentration varied from 1:48 to 1:400 but was usually in the neighborhood of 1:100. This was true even in the case of C. F. who, although he was given 500 mg. intravenously, voided only 60 cc. of urine two hours and again four and one-half hours after the injection, and who was later found to have urinary retention. He had a serum concentration of 2 $\mu\text{g./cc.}$ and urine concentrations of 200 and 100 $\mu\text{g./cc.}$, a ratio of 1:100 and 1:50.

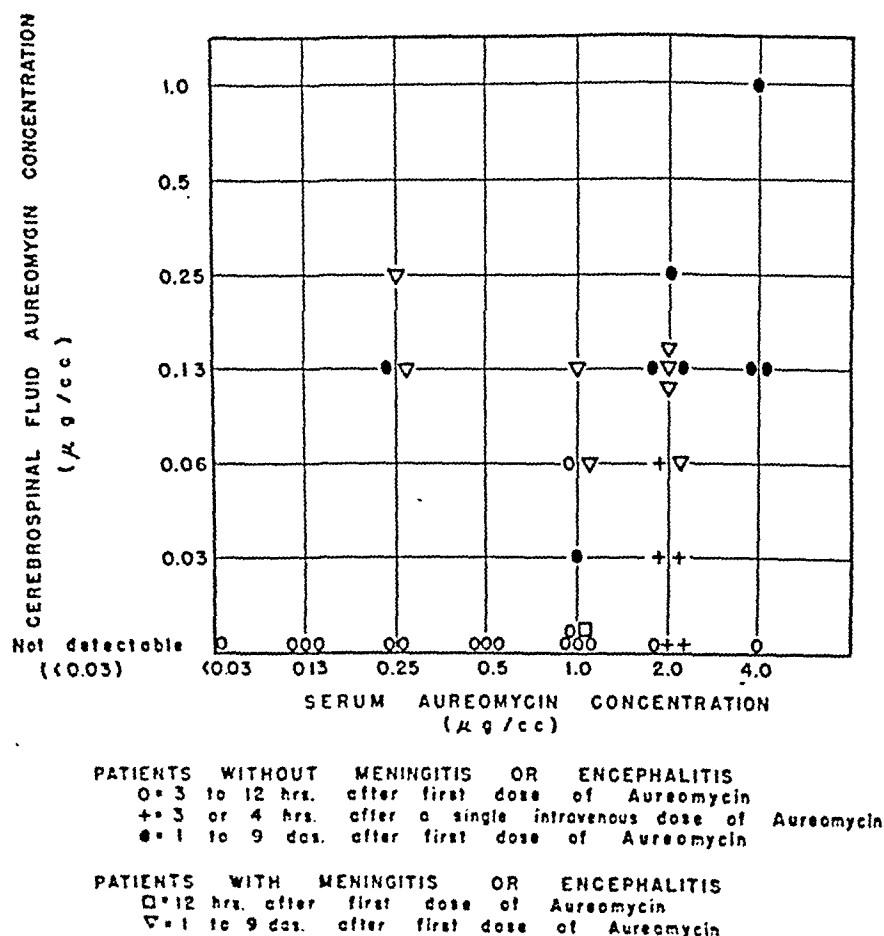


FIG. 1. THE CONCENTRATION OF AUREOMYCIN IN URINE AND CEREBROSPINAL, PLEURAL AND ASCITIC FLUIDS AFTER ORAL AND INTRAVENOUS ADMINISTRATION

TABLE II

Concentrations of aureomycin in urine and serum after oral and intravenous administration

Patient	Dose and route	Urine						Serum concentration	Serum/ Urine
		Hour collected	Volume	Concentration	Recovered	Per cent recovered			
						In time period	Cumulative		
E. P.	1 gm. P.O.	0-2	cc. 470	µg./cc. 64	mg. 30	3.0	3.0	µg./cc. 1.0	— — 1:128
		2-4	500	128	64	6.4	9.4		
		4-6	125	128	16	1.6	11.0		
		Total	1095						
N. B.	700 mg. P.O.	0-2	435	64	28	4.0	4.0	0.25 1.0	1:256 1:128 —
		2-4	475	128	61	8.7	12.7		
		4-6	150	128	19	2.7	15.4		
		Total	1060						
J. M.	300 mg. I.V.	0-2	175	12.5	2	0.7	0.7	0.25 0.125 0.125	1:50 1:400 1:48
		2-4	160	50	8	2.7	3.3		
		4-6	265	6	2	0.7	4.0		
		Total	600						
J. C.	300 mg. I.V.	0-2	60	100	6	2.0	—	1.0	1:100
W. B.	300 mg. I.V.	0-1	150	100	15	5.0	—	1.0	1:100
W. L.	300 mg. I.V.	0-1	65	100	7	2.3	—	1.0	1:100
J. B.	300 mg. I.V.	0-1	215	50	11	3.7	—	0.25	1:200
C. F.*	500 mg. I.V.	0-2	60	200	1	0.2	0.2	2.0	1:100
		2-4½	60	100	1	0.2	0.4	2.0	1:50

* Had prostatic hypertrophy and urinary retention.

DISCUSSION

It is apparent that after the first 24 hours of therapy with the doses of aureomycin in common use, detectable levels of the antibiotic are consistently present in the cerebrospinal fluid. Larger doses, producing higher serum concentrations, should result in higher cerebrospinal fluid levels. In some instances the antibiotic is present three hours after an intravenous injection of 500 mg. It is likely that higher intravenous doses might assure the presence of the antibiotic in the spinal fluid in all cases within a few hours. It is of interest that the one patient with pneumococcal meningitis whom we treated with oral doses of aureomycin recovered. The concentration of aureomycin in his cerebrospinal fluid was not detectable 12 hours after the start of therapy, but was 0.05 to 0.13 µg. thereafter. This patient received two doses of sulfonamides before aureomy-

cin was started. These may have prevented the progression of the meningitis before aureomycin was available at the site of the infection, although we doubt that they could have been responsible for the patient's recovery. In this connection, it is of interest that we have found the sensitivity of 26 strains of pneumococci to range between 0.01 and 0.16 µg. of aureomycin. Seventeen of the strains were sensitive to 0.02 µg. or less. Our preliminary studies show that aureomycin can be transmitted into the pleural and ascitic fluids when it is administered systemically. Whether this is true in all cases and whether the concentrations are sufficient for therapeutic effects are problems which await further study. The large volumes of urine obtained in some cases, and our observations on a number of patients under treatment with aureomycin, lead us to believe that aureomycin has a diuretic effect. The amount excreted is directly related to the serum concentration, the pH of

serum concentration to urine concentration being in the neighborhood of 1:100. This makes it likely that aureomycin is excreted as a glomerular filtrate. From our previous studies (1), which indicate that the serum concentrations are very low after 12 hours, and the fact that there is a constant serum-urine ratio, it can be deduced that the major portion of the aureomycin recoverable in the urine is excreted within 12 hours and that only relatively small amounts are recoverable thereafter. It would appear, therefore, that at most, only 15 to 20 per cent of the antibiotic will be recovered in the urine even after intravenous administration. Collins and his associates (3), measuring complete aureomycin excretion in two patients, found that 11 to 13 per cent was recovered after oral administration. The data available at present indicate, therefore, that the major portion of an intravenous dose of aureomycin is disposed of by methods other than urinary excretion and that the percentage recoverable in the urine is so small that the figure is of relatively little value in estimating the completeness of absorption from the gastro-intestinal tract.

SUMMARY

1. Among 17 specimens of cerebrospinal fluid obtained during the first 12 hours of oral administration, aureomycin was detectable in only one. When five patients were given an intravenous dose of 500 mg., detectable concentrations were present

in the cerebrospinal fluids of three patients within four hours. Aureomycin was present in all 16 specimens obtained 23 or more hours after the first oral dose, the concentrations ranging from 0.03 to 1 μ g. and being approximately related to the serum concentration present at the same time.

2. Aureomycin has been found in the pleural and ascitic fluids of two patients after oral administration.

3. Urine studies on eight patients showed an aureomycin concentration of 12.5 to 100 μ g./cc. during the first hour after oral or intravenous administration, depending upon the height of the serum concentration and the volume of urine excreted. The ratio of the aureomycin concentrations of serum to urine was approximately 1:100.

ACKNOWLEDGMENT

We wish to thank Mrs. Joan R. Broyhill, Miss Pearl Roberts and Miss Myrtle I. Meyer for technical assistance.

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CLINICAL EXPERIENCE WITH AUREOMYCIN^{1,2}

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During the past nine months a study has been made by members of the medical service at the Johns Hopkins Hospital concerning some of the possible therapeutic uses of aureomycin. Forty-eight patients with a variety of infectious processes were selected for treatment. In many cases the patients had been treated unsuccessfully with one or more chemotherapeutic agents before aureomycin was given but in each instance an effort was made to curtail other drugs during the period that aureomycin was used. Whenever possible the infectious agent was recovered before treatment with aureomycin was started, and other cultures were made during and following this therapy. The sensitivity to aureomycin of the bacteria recovered was tested *in vitro* in each instance.

Certain studies were made to evaluate the possible toxicity of the drug. The patients were examined daily for evidence of rashes or untoward symptoms. The blood and urine were examined before, during, and after treatment. The liver function of 37 cases was tested before and after treatment, by the thymol turbidity, cephalin flocculation, and BSP excretion tests and serial electrocardiograms were obtained from 39 cases. No patient developed drug fever or dermatitis, and no signs of toxicity to the bone marrow, kidneys, liver, or myocardium were noted. Twenty-six of the 48 patients complained of nausea or epigastric distress. Diarrhea was noted in addition in nine cases. The gastrointestinal symptoms seemed to depend on idiosyncrasy and some patients could take large doses without discomfort. In general, the same dose caused less discomfort when divided into small amounts given frequently rather than large amounts at wider intervals. Aureomycin was better tolerated when given with food or with

a buffer like Alugel but its effectiveness might be decreased by this procedure since it is known to deteriorate in alkaline environments. Many patients developed tolerance to aureomycin as treatment progressed. In only five cases was therapy stopped because of uncontrollable vomiting. It is of interest that four of these five were ambulant patients with sinusitis.

The patients have been divided into separate groups according to the diagnosis. The therapeutic results in each group will be considered separately.

Fifteen cases of urinary tract infection were treated. Six had chronic pyelonephritis, four had acute cystitis, three had post-operative infection with associated bacteremia, one had hydronephrosis, and one had a horse-shoe kidney. In 11 cases the infecting bacterium was *E. coli*, in two cases enterococcus, and in one, *Pseudomonas aeruginosa*. Each of the infecting strains of *E. coli* was inhibited *in vitro* by aureomycin in concentrations between 0.7 and 15 $\mu\text{g.}/\text{cc.}$ of medium. The strain of pseudomonas was resistant to 50 $\mu\text{g.}/\text{cc.}$ Fourteen of the 15 cases had not responded to other types of chemotherapy. The patients each received a total dosage of 4 to 32 gm. with an average of 13.2 gm. The results obtained were uniformly satisfactory except in the case of infection due to pseudomonas. All symptoms subsided within one to four days (average three and two-tenths days). In every instance the urine was sterilized. The follow-up periods are not yet very long but so far only the one patient with pseudomonas infection has had recurrence of the disease. Two other patients, following cystitis due to *E. coli*, developed stubborn infections due to proteus. This led us to avoid catheterization of other patients.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² Aureomycin supplied through the courtesy of Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

TABLE I

Primary atypical pneumonia treated with aureomycin

Case	No. of lobes	Agglutination		Day temperature reached normal			Aureomycin	
		Strept. MG	Cold	Disease	Hospital	Treatment	Grams	Days
JG	1	+	0	17	6	5	12.5	8
SK	1	+	0	6	4	4	16.0	7
ES	1	+	0	11	7	5	12.5	7
MF	1	+	0	11	5	4	7.0	4
FG	2	+	0	14	6	2	21.5	12
MF	2	+	+	7	5	4	12.5	7
Average				11.0	5.5	4.0	13.3	8.5

these patients recovered. The temperature reached normal on the second to fifth day of treatment (average four days). This corresponded with the 11th day of disease, or five and one-half days after hospitalization commenced. Nine additional patients with primary atypical pneumonia, admitted to the hospital during the study period, received no chemotherapy. The results are summarized in Table II. The first five listed were alternate admissions to the six treated cases. In this untreated series the results observed were just as satisfactory as in the treated cases. The temperature reached normal on the average after 10.1 days of disease and four and seven-tenths days of hospitalization. The results observed in these small groups of patients fail to indicate that aureomycin in the dosage employed affected the course of this disease. It seems obvious that larger groups of patients with adequate controls are necessary to settle this point.

TABLE II

Primary atypical pneumonia with no antimicrobial therapy

Case	No. of lobes	Agglutinations		Day temperature reached normal	
		Strept. MG	Cold	Disease	Hospital
AM	1	—	0	14	1
SR	1	—	0	12	1
MC	1	—	0	9	4
SM	1	—	0	5	3
IP	1	—	—	4	2
AC	1	—	0	7	3
ES	1	0	0	15	9
FL	1	—	—	8	6
WK	1	—	—	17	3
Average				10.1	4.7

Three patients with lung abscess were treated. Two had chronic infections for six and nine months, respectively. The third was acutely infected. The abscess in one patient was secondary to a pulmonary adenocarcinoma. From this case a pure culture of *E. coli* sensitive to 0.75 µg./cc. of aureomycin was obtained from the pus. No improvement had been noted following treatment with penicillin and sulfadiazine. After treatment with aureomycin, 4 gm. daily for 20 days, subjective improvement was noticeable and the temperature returned to normal. Subsequently, however, both fever and symptoms recurred and surgical drainage was necessary. It is of interest that the pus then contained no *E. coli* but instead a pure culture of proteus.

The second chronic case with a mixed infection resistant to penicillin and streptomycin was markedly improved after receiving aureomycin 4 gm. daily for six weeks. Fever and sputum disappeared and roentgenographic examination revealed a normal lung. The third patient had multiple acute abscesses due to staphylococcus and improved dramatically when treated with aureomycin although penicillin had seemed ineffective. Six patients with sinusitis were treated with satisfactory results.

An additional group of nine patients with different types of infections was treated with good results. These included two cases of lymphopathia venereum, and one case each of Rocky Mountain Spotted Fever, rat bite fever, tonsillitis due to *Streptococcus pyogenes*, meningitis due to *E. coli*, meningitis due to pneumococcus Type XIX, rodent ulcer, lobar pneumonia, and chronic brucellosis. One case of chronic ulcerative colitis showed marked symptomatic improvement on treatment.

Unsatisfactory results were observed in five additional patients comprising one case each of thyroiditis, pemphigus, sarcoid, osteomyelitis, and typhoid fever.

Four patients with bacterial endocarditis were treated. In two the infections, due to *Streptococcus fecalis*, developed following the Blalock-Taussig operation. The other two patients developed spontaneous infections due to microaerophilic streptococci. The courses of these four patients are summarized in Figures 1-4. In the case of K. S. (Figure 1) the *Streptococcus fecalis* was

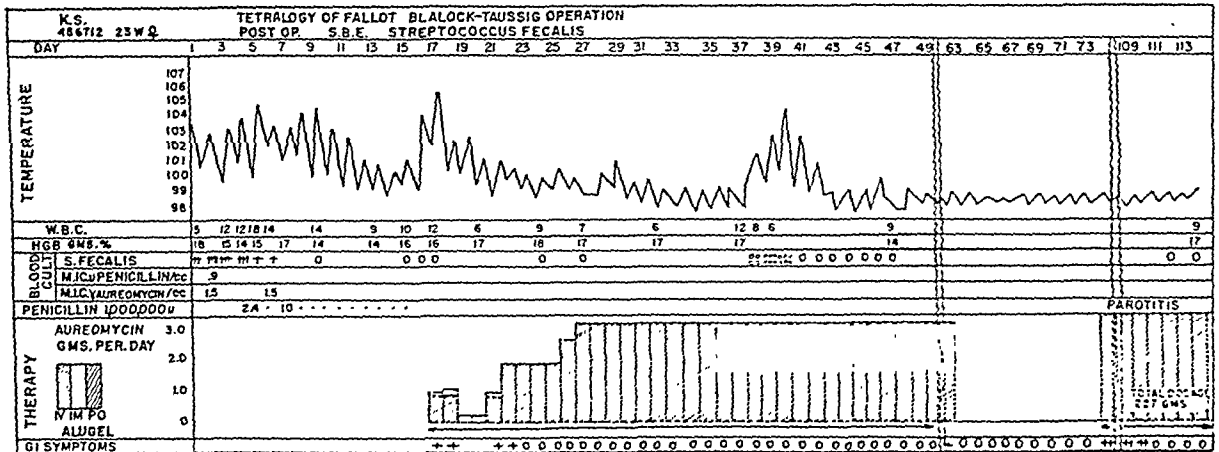


FIG. 1. SUBACUTE BACTERIAL ENDOCARDITIS, TREATED WITH AUREOMYCIN

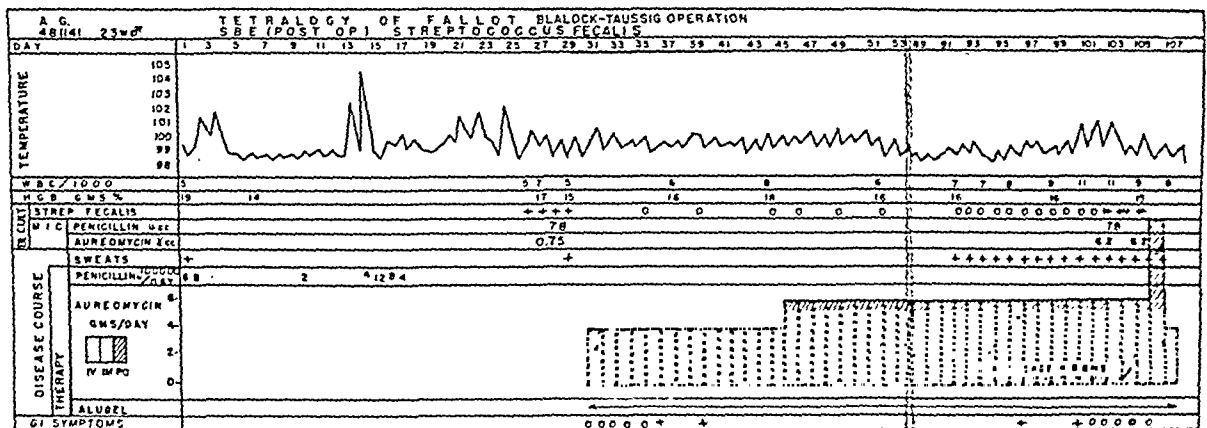


FIG. 2. SUBACUTE BACTERIAL ENDOCARDITIS, TREATED WITH AUREOMYCIN

sensitive *in vitro* to .9 u. of penicillin/cc. and 1.5 μ g. of aureomycin/cc. The patient was given 92 million u. of penicillin for 12 days when this was stopped because of the development of sensitivity to this drug. During penicillin therapy the blood

cultures had become sterile, and the patient showed definite clinical improvement although low grade fever continued. Following aureomycin therapy

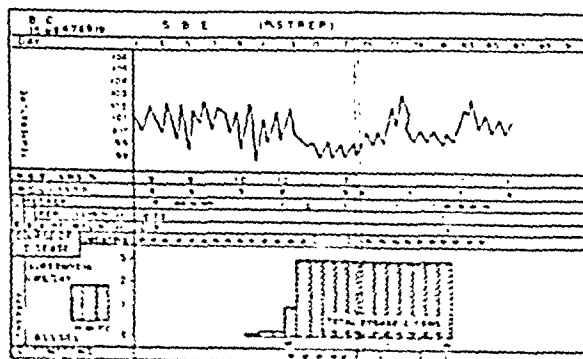


FIG. 3. SUBACUTE BACTERIAL ENDOCARDITIS, TREATED WITH AUREOMYCIN

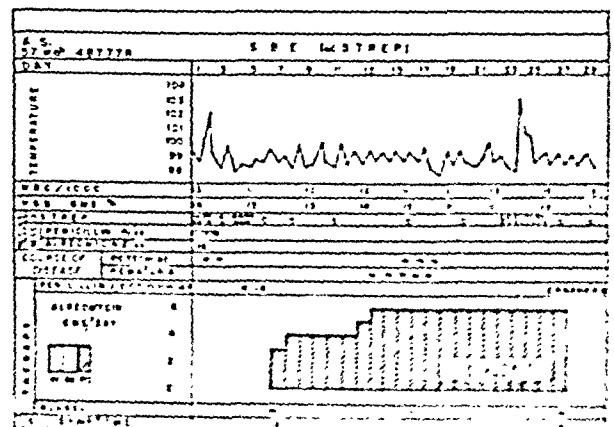


FIG. 4. SUBACUTE BACTERIAL ENDOCARDITIS, TREATED WITH AUREOMYCIN

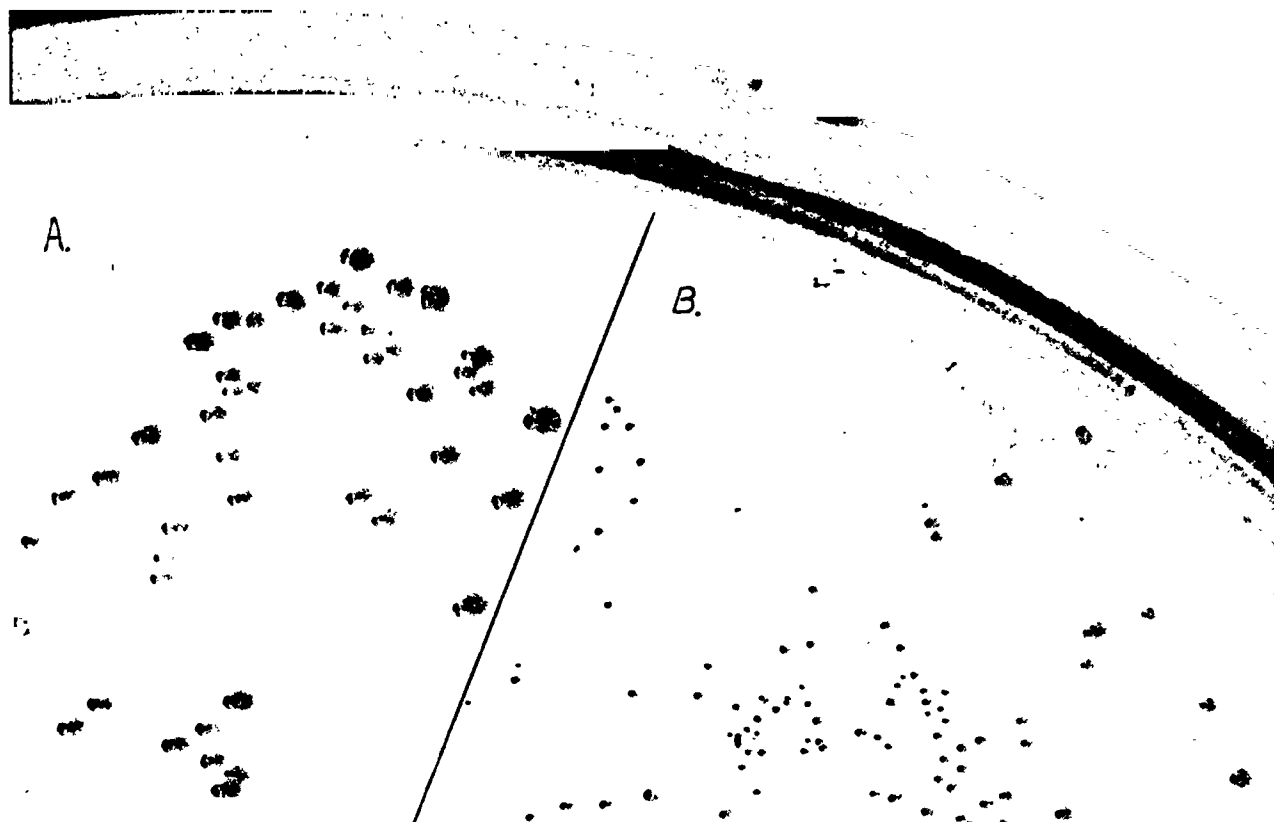


FIG. 5. COLONIAL FORMS OF *Streptococcus fecalis* GROWN ON AGAR PLATE CONTAINING BLOOD

A represents the large colonies formed by the strain recovered before treatment, inhibited by 0.75 $\mu\text{g./cc.}$ of aureomycin, but not less. B represents smaller colonies formed by the strain recovered after treatment with aureomycin, inhibited by 6.2 $\mu\text{g./cc.}$ of aureomycin, but not less.

TABLE III
Characteristics of *Streptococcus fecalis*—Strain AG

Characteristics	Strain No. 1 before therapy	Strain No. 2 after therapy
Sensitivity Aureomycin	0.75 $\mu\text{g./cc.}$	6.2 $\mu\text{g. cc.}$
Sensitivity Penicillin	7.8 u. cc.	7.8 u./cc.
Colonies Blood agar	large, translucent	small, more opaque
Growth— T.S. broth	6 hr.—500,000,000/cc. 24 hr.—1,500,000,000 cc.	6 hr.—50,000,000 cc. 24 hr.—50,000,000/cc.
Morphology	Large, elongated G+ cocci in pairs, groups, and short chains	Same
Growth in broth	Smooth	Smooth
Heat resistance 62° C.—30 min.	—	+
Litmus milk	Reduced, coagulated, peptonized	Same
Liquefaction serum clots	Liquefied	Liquefied
Liquefaction serum clots (after treatment)	—	—
Optimal growth pH	6.5	7.0
Substrate utilization	Hydrolyzed	Hydrolyzed

the temperature became normal, clinical improvement was progressive, and the patient remains well five months since discharge from the hospital. In the other three cases, evidence of infection persisted during or subsequent to the aureomycin treatment.

It is of interest to note that the strains of streptococci recovered from the blood stream of cases B. C. and A. G. after aureomycin treatment showed greater resistance to the bacteriostatic effect of aureomycin *in vitro* than the original strain obtained before treatment. The increases noted were 16 fold and eight fold, respectively. Detailed studies were made of the strain obtained from A. G. It was noted that the resistant strain grew more slowly in broth and the colonies on agar plates containing blood were smaller than those of the original sensitive strain. All the other characteristics tested were identical. These observations are shown in Table III and Figure 5. The changes in growth rate and colonial form are

suggestive of those described by Eriksen (1) for staphylococci resistant to penicillin. The small resistant variants of this streptococcus were unstable and reverted quickly to the fast growing aureomycin sensitive type.

It is quite obvious that in each case the drug has some therapeutic effect in that the temperature became normal and blood cultures became sterile. The reasons for the subsequent reappearance of the bacteremia in two of the cases infected with organisms quite sensitive *in vitro* are not now obvious. The development of increased resistance as illustrated in the two cases B. C. and A. G. may have played a part in the ultimate failure in therapy but there may be other equally important

factors such as the inability of aureomycin to penetrate the vegetations.

In summary, aureomycin is a non-toxic drug which may be useful in a variety of infectious diseases. In our experience the results in urinary tract infections were particularly good. The relatively slow deterioration of aureomycin in acid environments may contribute to its effectiveness in this type of infection. The use of aureomycin in combination with other antimicrobial agents was not tested.

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THE CLINICAL EVALUATION OF AUREOMYCIN^{1, 2, 3}

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Aureomycin (1), an antibiotic agent derived from *Streptomyces aureofaciens*, has been demonstrated to have therapeutic activity against a wide variety of bacterial, rickettsial, and viral pathogens. *In vitro* and *in vivo* activity has been observed against most gram-positive cocci, and gram-negative cocci and rods (1-6). In addition, *in vivo* activity, has been shown against the rickettsiae of the typhus, Rocky Mountain spotted fever, scrub typhus, and Q fever groups, as well as against the viruses of lymphopathia venereum and psittacosis (7, 8). Animal toxicity has been noted to be very slight (9).

Successful clinical trials have been reported in a wide variety of bacterial and rickettsial infections, as well as infections due to lymphopathia venereum virus and the causative agent of primary atypical pneumonia (4, 6, 10-24). Toxic reactions have been few and insignificant. Since the number of patients reported with each type of infection has been small, and since all patients were treated by the oral or intramuscular routes, we are documenting the results of clinical trial of aureomycin in 116 patients observed since May, 1948. Many of these patients received aureomycin by the intravenous route. Assays of serum concentrations of aureomycin were carried out in most patients by means of a method previously described (25). *In vitro* sensitivity tests on the infecting organisms were carried out wherever pos-

sible. Studies on the clinical pharmacology of aureomycin are being presented elsewhere (26).

MATERIALS AND METHODS

The majority of the patients represented in the present report were treated with aureomycin while hospitalized at the San Francisco Hospital or the University of California Hospital. In several instances, the patients were treated in other locations under the guidance of one or more of the present investigators, and laboratory studies were carried out under their direction. Most of the viral and rickettsial isolations and serological procedures were carried out at the State of California Virus and Rickettsial Disease Laboratory. The assays of serum aureomycin concentration were carried out at the Infectious Disease Laboratory, as were determinations of the aureomycin sensitivity of the infecting bacterial organisms (Tables Ia and Ib).

All patients were accepted for inclusion in the investigative series only when adequate clinical and laboratory criteria for diagnosis were satisfied.

Dosage and Route of Administration

Adult patients in this series taking aureomycin by mouth generally received 1 gram every four to eight hours. Most patients received 1 gram every six hours. In many infections this dosage schedule may prove to be excessive. Several patients suffering from primary atypical pneumonia have responded satisfactorily to 0.5 gram every six hours, and 0.5 gram every eight hours seemed adequate in one patient whose urinary tract infection was due to a moderately susceptible organism. On the other hand, large doses may be required to produce satisfactory results in patients infected with resistant bacteria such as *Pseudomonas aeruginosa* and *Proteus vulgaris*, in typhoid fever, and in Q fever.

Sixty-eight⁵ individuals received 337 intravenous injections of aureomycin. In 327 instances the aureomycin was dissolved immediately before injection in a solution containing 131 mg. of *l*-leucine per 5 cc. A concentration of 10 mg. of aureomycin per cc. was used. A 0.784 per cent sodium carbonate solution appeared to be a satisfactory diluent in 10 intravenous injections. As many as 36 injections of 100 mg. were given in an individual patient without untoward result. The individual intravenous dose varied from 40 to 200 mg. Intravenous doses of 50 to 100 mg. have been demonstrated to produce serum levels of from 2.5 to 10 μ g. five minutes after

⁵ This figure includes 12 normal individuals who received intravenous aureomycin in the course of pharmacological experiments.

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³ The aureomycin used in this investigation was provided by Lederle Laboratories, Division of the American Cyanamid Company.

⁴ This laboratory is supported by the Divisions of Medicine and Pediatrics of the University of California Medical School and by the Department of Public Health of the City and County of San Francisco, California.

injection (25, 26). Concentrations of aureomycin in excess of 0.15 $\mu\text{g. per cc.}$ usually persisted from six to eight hours. Combined simultaneous oral and intravenous treatment resulted in serum concentrations as high as 20 to 80 $\mu\text{g. per cc.}$

Intravenous medication was used according to two general plans:

1) One or two intravenous doses were given initially, often combined with oral doses, to achieve an immediate high blood concentration of aureomycin. High serum levels of aureomycin often did not appear for one or more days when only oral administration was used. These initial priming doses were followed by maintenance on oral therapy (32 patients).

2) Three intravenous doses, usually of 100 mg., were administered at six-hour intervals during the day supplemented by one oral dose at night during the entire course of treatment (22 patients). Two patients were treated satisfactorily with three intravenous injections daily without supplementary oral medication.

The therapeutic effects of intravenous medication did not appear to differ from those following from oral administration alone in infection due to susceptible agents. Treatment by the intramuscular route was abandoned following the very frequent occurrence of painful local reactions and the demonstration that measurable serum concentrations of aureomycin rarely resulted.

While the relationship of *in vitro* sensitivity tests of infecting organisms and optimum serum aureomycin concentrations to produce favorable clinical response is as yet unknown, such *in vitro* tests in this laboratory partially helped clarify in which types of infection success or failure would be likely to occur.

In this laboratory most strains of gram-positive cocci and *C. diphtheriae* were sensitive to less than 0.1 $\mu\text{g. per cc.}$, according to the method used. Most strains of *E. coli*, *A. aerogenes*, *E. typhosa*, *Salmonellae*, *N. meningitis*, *K. pneumoniae*, *H. influenzae*, and *Shigellae* were sensitive to less than 1.0 $\mu\text{g. per cc.}$ *Pr. vulgaris* and *Ps. aeruginosa* were found to be susceptible only to from 3.5 to 288 $\mu\text{g. per cc.}$ (26). Spontaneous development of aureomycin resistance following unsuccessful treatment was observed in only one instance, that of a urinary tract infection due to *E. coli*. In this patient the infecting organism was sensitive to 0.21 $\mu\text{g. per cc.}$ before the beginning of treatment, while an *E. coli* recovered during relapse was inhibited only by 285 $\mu\text{g. per cc.}$

Toxicity

No serious toxic effects of aureomycin were noted. Nausea and/or vomiting occurred in more than one-half of patients receiving oral doses of 1 gram every four or six hours. Occasionally this was sufficiently severe to prevent further treatment by this route. That this distressing symptom was principally local in cause was evidenced by the fact that only two of 68 patients receiving aureomycin intravenously experienced nausea and in neither instance was the symptom certainly attributable to medication. Patients receiving three intravenous and one oral dose per day did not experience significant

nausea. On several occasions positive guaiac reactions for occult blood were observed in the stools of patients receiving aureomycin by mouth. Nausea occasionally subsided spontaneously in spite of continuation of oral therapy. Aluminum hydroxide gels at times appeared to give relief, as did sedative and antispasmodic medications. Frequent or loose stools were commonly observed but did not prevent continuation of therapy. The serum concentration of aureomycin bore no relationship to untoward gastrointestinal manifestations.

Maculo-papular, urticarial, or scarlatiniform rashes were observed in six patients. In two of these the rash could have logically been ascribed to some other cause than aureomycin medication. Drug fever was considered a possibility in two instances. Vertigo occurred in two patients receiving aureomycin intravenously after only short periods of treatment and in the absence of unusually high blood concentrations. The significance of this observation is uncertain. "Black tongue" occurred in two patients. Two instances of glossitis in association with rashes were noted. Local spasm of veins during injection was observed very rarely. No sloughs resulted from extravasation outside the vein. Erythema and pruritus of the perineal area occurred in four patients. Intramuscular injection of aureomycin dissolved in Sorenson's phosphate buffer (pH 7.4) and combined with 2 per cent procaine almost invariably resulted in severe, painful, local reactions.

RESULTS

Typhoid, Salmonella and Shigella Infections

In this group of patients, the results following aureomycin therapy were variable, and for the most part could not be interpreted as showing a therapeutic effect of the drug. However, in certain cases the results were such as to convince one that the drug had been of specific clinical value.

In one of two carriers of *E. typhosa* who had no clinical disease, there was temporary absence of the organism during aureomycin therapy. The second carrier showed no change in the stool culture for *E. typhosa*. In a third patient who developed a biliary fistula following cholecystectomy and who showed the presence of *E. typhosa* in both feces and in the discharge from the fistula, there was healing of the fistula and abolition of the fecal carrier state following aureomycin therapy. Long-term follow-up of this case has not been completed at this time.

In nine cases of acute typhoid fever, only two showed a response to aureomycin therapy. The first of these patients, L. W., whose course is summarized in Figure 1 and given in detail below, was a 21-month-old child who was started on aureo-

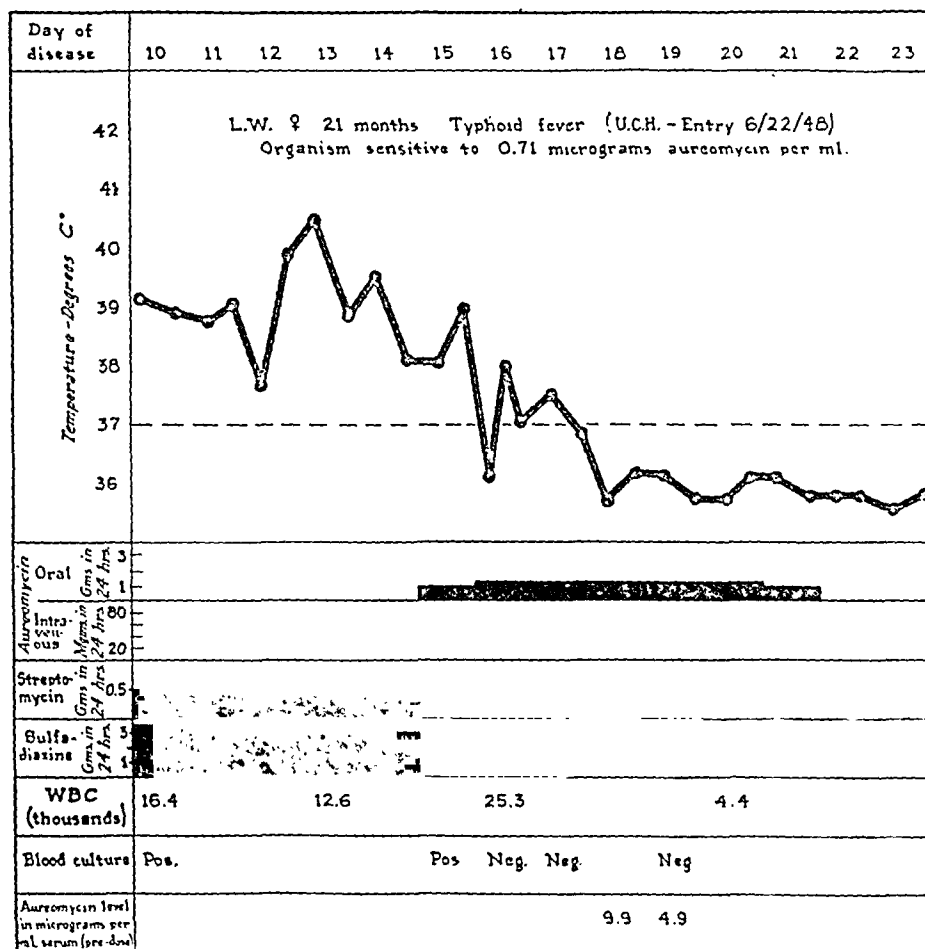


FIG. 1

mycin on the 16th day of fever at which time the blood culture was positive for *E. typhosa*. She was given 1.0 gram of the drug daily and her temperature was within normal range in 48 hours. Her blood culture became sterile the day after therapy was started.

Case 1. L. W., white, female, age 21 months. This infant entered the University of California Hospital on June 22, 1948. On June 14 she had developed chills, fever and prostration which had continued unabated until hospital admission. A blood culture taken before entry proved to be positive for *E. typhosa*.

Physical examination revealed the rectal temperature to be 39.4° C. The child appeared severely ill and was irritable and uncooperative. No other positive physical findings were noted.

Urinalysis was negative. Complete blood count showed: Hemoglobin 11.0 grams per cent; red blood cells 4,100,000 per cubic mm.; white blood cells 16,000 per cubic mm.; neutrophils 74 per cent; lymphocytes 20 per cent; monocytes 6 per cent. Blood cultures taken on June 24 and June 27 were positive for *E. typhosa*. Stool cultures on July 2 and July 7 were negative. Urine culture on June 22 revealed only contaminating *E. coli*.

No clinical or bacteriological response followed administration of streptomycin and sulfadiazine from June 23

to June 27. On June 27 aureomycin therapy was begun. The patient received 0.16 gram by mouth every four hours until July 3. The temperature became normal in 48 hours and simultaneous general improvement was noted. Blood culture 24 hours after the institution of aureomycin was negative and the blood stream remained sterile thereafter. On July 2 and 3 an urticarial rash appeared over the trunk and faded spontaneously. The child had previously had urticaria after ingestion of several different foods. The possibility of aureomycin sensitivity remained uncertain. Further convalescence was uneventful.

The other patient showing a favorable response was also a child, age 22 months, who was started on 1.0 gram per day in four daily doses on the 13th day of disease at which time the blood culture was positive for *E. typhosa* and the temperature was 104° F. Her temperature dropped to below 100° F. within 48 hours and was within normal range within four days. There was marked symptomatic improvement within 48 hours.

Another patient was treated for a short time with aureomycin and then chloromycetin therapy was instituted. During aureomycin therapy the

TABLE I-A

Summary of the results of aureomycin treatment in bacterial infections

Disease	Total no. of cases	Results		
		Good	Equivocal	Poor
Urinary tract infections	13	10*	1	2
Brucellosis	3	2	—	1
Chancroid	4	4	—	—
Typhoid fever	9	2	3	4
Typhoid carrier	3	—	1	2
Salmonellosis	4	1	3	—
Shigellosis	2	2	—	—
Leptospirosis	1	1	—	—
Pneumonia, pneumococcal	2	1	1	—
Pneumonia, staphylococcal	1	—	1	—
Septicemia, pseudomonas	1	—	—	1
Erysipelas	1	1	—	—
Subacute bacterial endocarditis, <i>Streptococcus fecalis</i>	2	—	2	—
Peritonitis, generalized	2	2	—	—
Pelvic abscess— <i>E. coli</i>	1	—	1	—
Post-operative fecal fistula	1	—	—	1
Pyoderma—staphylococcal	1	1	—	—

* Five patients had recurrences or reinfection.

blood culture became negative but the temperature course was unchanged. Two days after chloromycetin was started the temperature had dropped to within normal range and remained there. This case is summarized in Figure 2 and given in detail as follows:

Case 2. N. S., white female, age 43 (SFH 74120). Two weeks before admission to the San Francisco Hospital on March 9, 1949, this patient had noted the sudden onset of fever following the extraction of an abscessed tooth. She was admitted to another hospital where she had had slight diarrhea. Her blood culture was found to be positive for *E. typhosa* and her Widal test was

TABLE I-B

Summary of the results of aureomycin treatment in rickettsial, viral and miscellaneous infections

Disease	Total no. of cases	Results		
		Good	Equivocal	Poor
Q fever	29	18	4	7
Primary atypical pneumonia	16	13	3	—
Psittacosis	3	3	—	—
Herpes zoster	1	1	—	—
Varicella	2	—	1	1
Lymphopathia venereum	1	1	—	—
Infectious mononucleosis	3	—	3	—
Pneumonia—undetermined etiology	6	2	2	2
Pemphigus	3	—	2	1
Primary coxidioid pneumonia	1	—	—	1

positive in a dilution of 1:320. She was then transferred to the San Francisco Hospital.

Past history was irrelevant. Examination at the time of admission revealed the temperature to be 40° C., pulse 100 per minute, respiration 24 per minute. The patient appeared toxic and lethargic, but was readily aroused. Numerous fresh rose spots were present on the abdomen. The spleen was not palpable. Physical examination was otherwise negative.

Urinalysis revealed one-plus albuminuria. Complete blood count showed: red blood cells 4,200,000 per cubic mm.; packed cell volume 34 per cent; white blood cells 3,800 per cubic mm.; neutrophils 66 per cent; eosinophils 2 per cent; lymphocytes 32 per cent. Blood culture was positive for *E. typhosa*. Urine and stool cultures were repeatedly negative for pathogens. Agglutinations with typhoid H and O antigens were both positive in a titer of 1:160. Chest X-ray was negative.

On March 11 the patient was started on aureomycin. She received 100 mg. intravenously every six hours. One oral dose of 1 gram was substituted for the night intravenous dose. Aureomycin was continued until March 14. Although the blood culture had become negative by March 12, no effect on the temperature or condition of the patient was apparent. On March 14 the patient was begun on chloromycetin. She received 1 gram every six hours by mouth until March 16 when the dose was changed to 0.5 gram every three hours by mouth. By March 17 the temperature had become normal and the patient appeared improved. She remained afebrile thereafter. Chloromycetin was discontinued on March 21.

In four of the remaining patients with acute typhoid fever, aureomycin therapy seemed to have caused a slight effect on the fever curve which could be interpreted as a temporary suppression of the infecting organism. In these patients the therapy caused no change in the course of the disease.

In four cases of infection due to organisms of the *Salmonella* group, unequivocally good results were obtained in only one, that of a septicemia without enteritis due to *Salmonella schottmulleri*. In this case, that of a 15-month-old male, penicillin, streptomycin, and sulfadiazine had had no effect on the blood culture which became negative 48 hours after starting aureomycin. The temperature came to within normal range after a similar interval. The possibility that this apparent response to the drug represents a spontaneous recovery is admitted but the rapidity of the temperature response led us to conclude that it was probably a therapeutic effect.

In three cases of enteritis due to *S. enteritidis*, *S. typhimurium* and *S. sch. mulleri* a similar re-

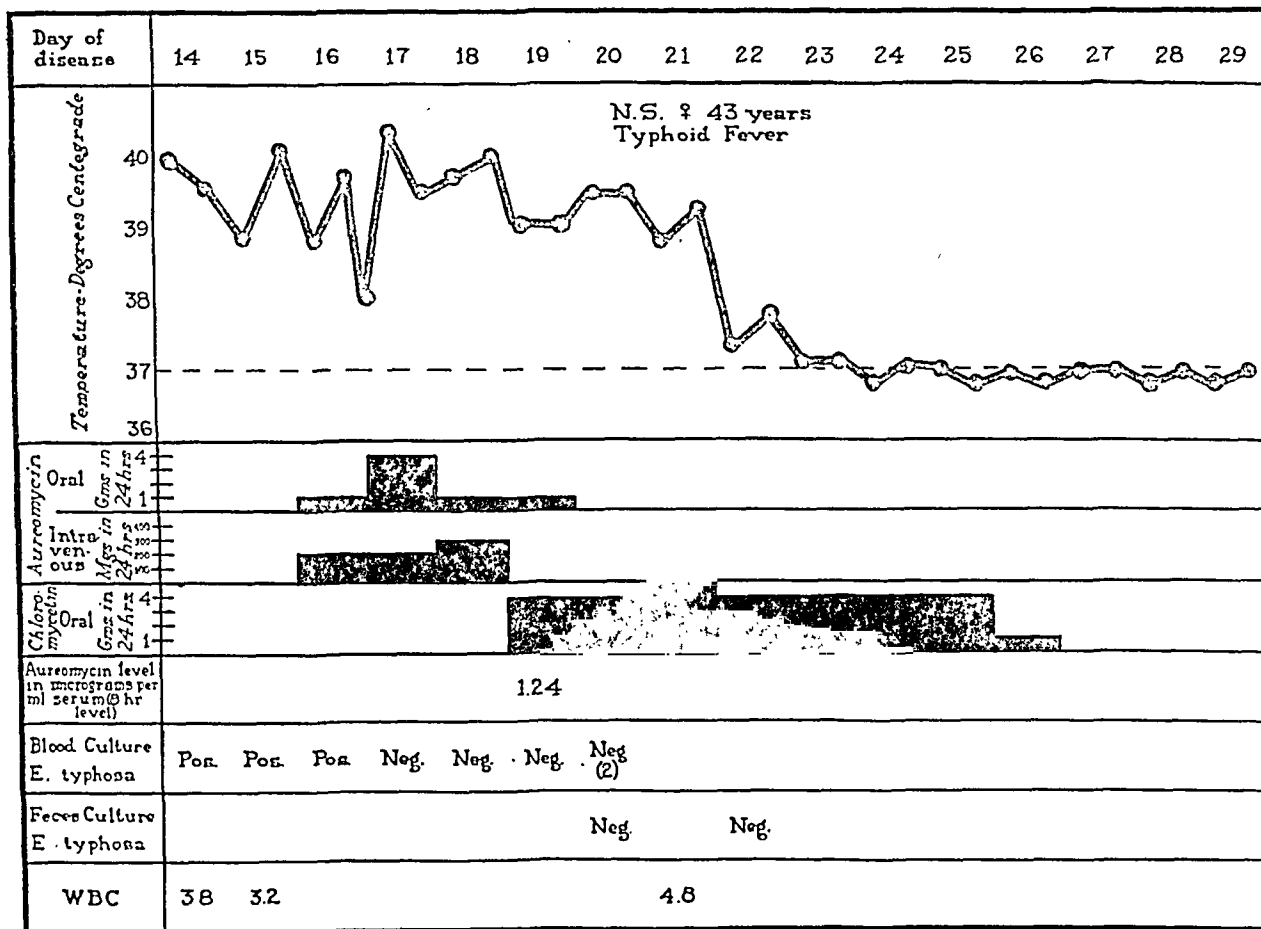


FIG. 2

sults were noted. *S. enteritidis* and *S. schottmulleri* were not found in the stool cultures during treatment but promptly reappeared when therapy was stopped. In the case of *S. typhimurium*, the organism was not found in the stool after starting aureomycin and there was no recurrence.

Two cases of *Shigella* infections have been

treated, both with apparently good immediate results. A four-year-old girl with dysentery due to *Shigella* showed prompt bacteriological and clinical response with no recurrence following cessation of therapy. In the second case, a four-year-old boy with dysentery due to *Shigella flexner*, the stool cultures were free of this organism 24 hours after

TABLE II
The effect of aureomycin on infections of the urinary tract

Patient	Infecting organisms	Anatomical lesions	Result
L. A.	<i>A. aerogenes</i>	None	Rapid sterilization
R. I.	<i>Strep. fecalis</i> , <i>E. Coli</i> , <i>P. vulgaris</i>	None	Rapid sterilization
T. Y.	<i>Staph. aureus</i> , <i>Strep. fecalis</i>	None	Rapid sterilization
S. U.	<i>P. vulgaris</i>	Pyelectasis	Rapid sterilization
B. A.	<i>Ps. aeruginosa</i> , <i>Staph. aureus</i>	Ureteroplasty	Sterilization; later recurrence of both organisms
S. E.	<i>Ps. aeruginosa</i> , <i>A. aerogenes</i>	Possible vesico-vaginal fistula	Sterilization; later recurrence of pseudomonas
R. E.	<i>Ps. aeruginosa</i>	Ureteroplasty	Sterilization; later recurrence
F. E.	<i>Ps. aeruginosa</i> , <i>Strep. fecalis</i>	Urethral valve	Sterilization; later reinfection with <i>E. coli</i>
Y. O.	<i>Ps. aeruginosa</i>	Hydronephrosis	Sterilization; no follow-up
C. O.	<i>E. coli</i>	Hydronephrosis	Sterilization; recurrence with resistant variant
B. R.	<i>Ps. aeruginosa</i>	Hydronephrosis	Sterilization; no follow-up
C. A.	<i>Ps. aeruginosa</i>	Hydronephrosis and calculi	Suppression and disappearance of fever
E. C.	<i>Ps. aeruginosa</i>	Hydronephrosis	No effect; received only 1.5 grams orally
G. F.	<i>Ps. aeruginosa</i>	Cord bladder	No effect; received only 100 mg. intravenously

starting aureomycin but the diarrhea continued for three more days. Long term bacteriological follow-up of this second case has not been completed.

While the total number of cases of infection due to organisms of the Typhoid-Salmonella-Shigella group is not large, it is probably a safe conclusion that, in our hands, aureomycin has been of doubtful value in most cases.

Infections of the Urinary Tract (Table II)

Aureomycin has been reported as favorably affecting infections of the urinary tract by others (14, 17, 24). Fourteen individuals with urinary tract infections, in all instances previously treated unsuccessfully with sulfonamides and other antimicrobial agents, were selected for therapy with aureomycin. In 11 of these patients chronic infection was certainly or probably associated with obstruction of the urinary passages. Three patients suffered from acute non-obstructive infections. All of the latter group responded quickly by sterilization of the urine and disappearance of symptoms. While temporary sterilization of the

urine was accomplished in 10 patients in the former group, excepting two who received very small amounts of aureomycin, relapse or reinfection occurred in all patients in whom follow-up cultures were obtainable. In only one instance was decrease in the sensitivity of the organism to aureomycin isolated during relapse observed. It was felt that aureomycin controlled the infection in the instances of hydronephrosis of pregnancy so that the fetus could be carried to term. In one instance pyelolithotomy was successfully accomplished during a period of suppression of infection by aureomycin. Infections due to *Pseudomonas aeruginosa* proved to be the most difficult to treat successfully.

Brucellosis

Three cases of brucellosis have been treated with aureomycin in the present investigation. Spink (18) has reported on 24 cases of brucellosis treated with aureomycin with favorable results.

The first case in the present report was that of a 21-year-old female who was treated for a period of 23 days starting on about the 63rd day of the

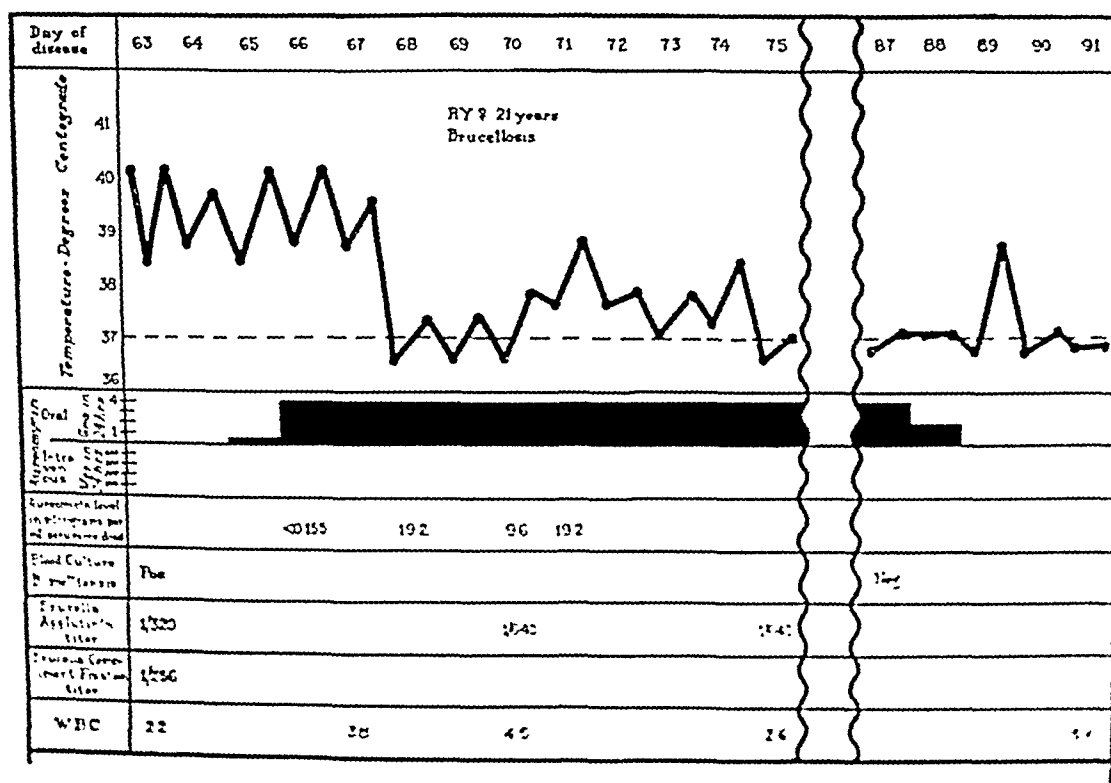


FIG. 3

disease. Her response was very satisfactory and the course is summarized in Figure 3 and a detailed case history follows.

Case 3. R. Y., white, female, age 21 (SFH 39899). The patient entered San Francisco Hospital on December 16, 1948, stating that she had had progressively severe bouts of nocturnal fever and sweating accompanied by joint pains for six weeks. In addition she had developed frequent nausea and vomiting and occasional epigastric pain during these episodes. Six weeks previous to the onset of her illness she had returned from several months' stay in Mexico. Her past history was irrelevant.

On examination the temperature was found to be 38.4° C., pulse 120 per minute, respiration 21 per minute. The patient appeared severely and chronically ill. There was slight generalized lymphadenopathy. The liver edge was palpated 3 cm. below the right costal margin. The spleen extended 7 cm. below the left costal margin. Physical examination was otherwise essentially negative.

Urinalysis revealed minimal albuminuria. Complete blood count showed: Hemoglobin 10 grams per cent; red blood cells 3,410,000 per cubic mm.; white blood cells 1,450 per cubic mm.; neutrophils 64 per cent; lymphocytes 38 per cent. Corrected sedimentation rate (Wintrobe) was 17 mm. per hour. Stool was negative for occult blood and pathogens. Chest X-ray, tuberculin and coccidioidin skin tests were negative. Agglutinations for typhoid and paratyphoid were repeatedly negative. Complement fixations for Q fever and psittacosis were negative. Brucella agglutinins were present in a titer of 1:320 on December 23, 1948 and gradually rose to 1:1,280 on January 10, 1949. After repeatedly sterile blood cultures, *Brucella melitensis* (type H2s) was isolated on January 3, 1949. On January 5 the patient received 50 mg. of aureomycin orally every six hours. On the following day this was increased to 250 mg. The patient received 1 gram every six hours from January 7 to January 28, and then 0.5 gram every six hours until January 30. Rapid defervescence occurred within 48 hours although the patient continued to run a low-grade fever until January 22. Symptomatic improvement coincided with the febrile response. On January 29 the patient had a chill followed by a transient febrile rise and was noted to have a macular rash over the entire body which faded rapidly by the next day. This patient has since remained entirely well.

The second case of brucellosis was that of a 32-year-old man whose treatment was started on the 15th day of disease and who received 4 grams per day for 13 days. The diagnosis in this case was based on a rise in Brucella agglutinin titer of from 1:500 on the sixth day of disease to 1:2,560 on the 13th day of the disease. His temperature, which had ranged around 100° to 103° F. for 15 days, fell to within normal limits within 48 hours and remained there.

The third individual, a 39-year-old woman who had had chronic brucellosis for four years, received 4 grams per day for 13 days after a preliminary three-day period during which she received 250, 500, and 750 mg., respectively. Amelioration of subjective symptoms was marked and almost immediate. Her expected bouts of low-grade fever did not occur during treatment or for two weeks afterward. At that time she again developed malaise and occasional low-grade fever. It was considered that aureomycin had probably exerted temporary suppressive effect on the infection.

Leptospirosis

Heilman (27) reported that aureomycin exhibited greater therapeutic effect on experimental infection with *Leptospira icterohemorrhagica* than did penicillin. A single severely ill patient was treated with aureomycin administered intravenously and orally from the sixth to 17th day of illness. Prompt amelioration of the symptoms appeared to result. The blood urea declined from 120 mg. per cent to 24 mg. per cent within one week. Liver function returned to normal more slowly. Although the rapid improvement could not certainly be attributed to aureomycin, it was felt that specific anti-spirochaetal effect was most likely. This case is presented in detail.

Case 4. J. J., white, male, age 42 (SFH 5389). This patient was brought to the San Francisco Hospital in an unconscious state on January 26, 1949. His wife stated that he had developed fever, chilly sensations, and malaise four days previously. He complained of severe aching in the shoulders, back and knees. He lost his appetite but did not vomit. His skin became yellow and his vision was blurred. On the morning of entry his speech became confused. Shortly thereafter he had three generalized convulsions and lapsed into coma. The patient was a sewer worker and had frequently encountered rats.

On examination his temperature was 37.8° C., pulse was unobtainable, respiration 22 per minute. He was completely unconscious. The skin and sclerae were icteric. Clotted blood was present in the right naris. The liver dullness extended three finger-breadths below the right costal margin. The spleen was not palpable. The reflexes were slightly more active on the right. During the 24 hours following hospital admission numerous subconjunctival hemorrhages appeared and purpuric spots were noted in the skin. The patient became semi-conscious but remained confused.

Urinalysis revealed mild albuminuria and a few hyaline and granular casts. Bile was absent. Hematological studies showed: packed cell volume—35 per cent; white

TABLE III

Liver and kidney function in patient with leptospirosis treated with aureomycin

Date	Jan. 26	Jan. 27	Jan. 28*	Jan. 31	Feb. 2	Feb. 3	Feb. 5	Feb. 15	Feb. 25
Icteric index, units	125	135	150	163	132	—	50	24	17
Blood urea, mg. per cent		120	75	21		52	24		
Cephalin flocculation	+++	+++	+++	++++					

* Treatment started

blood cells 13,650 per cubic mm.; neutrophils 87 per cent; lymphocytes 13 per cent. A spinal puncture revealed a xanthochromic fluid containing 6,250 red blood cells per cubic mm. On January 28 *Leptospira* were demonstrated by darkfield examination of the blood. Agglutinins for *L. canicola* were present in a dilution of 1:100. No agglutinins for *L. icterohemorrhagica* were present. Darkfield examination of the blood on January 29 was again positive. Agglutinins for both *L. canicola* and *L. icterohemorrhagica* were present in a titer greater than 1:10,000 on February 4. Complement fixation reactions for Q fever and psittacosis were negative.

On January 28 the patient was begun on aureomycin therapy. He received 1 gram orally every six hours until February 8. In addition, 100 mg. were administered intravenously three times daily during the first five days.

The patient's sensorium cleared rapidly and his appetite and strength returned. The icteric index and blood urea were gradually decreased. The patient had little fever at any time after hospital admission. (See Table III.)

Q Fever

Twenty-nine patients, proven by serologic or animal tests to have Q fever, have been treated to the present time. A large number of these cases have been presented in a separate report (13). In 19 patients the drug was given orally. Two were treated by intramuscular injections and seven patients were treated by both the oral and intramuscular routes. One individual has been treated by the combined oral and intravenous routes.

The two patients treated by the intramuscular route alone recovered quickly but no conclusions can be drawn. They were treated early in this study and with what is now considered an inadequate dosage.

Fourteen of the 19 patients receiving aureomycin by the oral route alone were treated within seven days of onset of their illness. Treatment in the remaining four patients was begun on the 10th, 13th, 17th, and 23rd days, respectively, after onset of illness. Six patients became afebrile within 48 hours after institution of therapy and seven within

72 hours. Fever persisted in three patients for five days, however, in one for eight days, and in one for 13 days after beginning of treatment. The remaining individual had suffered an attack of Q fever two months previously and had continued to run a low-grade fever. Because of this persistent fever, he was treated with aureomycin orally for nine days and became afebrile on the first day of treatment. His fever did not recur and there was marked subjective improvement.

Therapy by the oral and intramuscular routes in six patients was begun on the third, fourth, seventh, tenth, 14th and 27th days respectively, after onset of illness. Temperature returned to normal in these six patients within 25, 26, four, 25, five and 18 days, respectively. Four of these patients were severely ill and large amounts of the antibiotic were given; because of severe nausea three of these individuals tolerated the drug poorly. The seventh patient in this group had run a prolonged convalescence with low-grade fever following an acute attack of Q fever nine weeks before; he became afebrile on the first day of treatment and remained afebrile.

One severely ill individual was treated by the combined oral and intravenous routes. He received a total of 2,100 mg. parenterally and 40.0 grams orally in two courses over a period of eight days. Treatment was begun on the 16th day of illness; 29 days later the patient remained febrile but improved, and the drug appeared to have had little effect on the course of the illness.

Primary Atypical Pneumonia

In the present investigation there were 16 patients treated with aureomycin whose clinical course and physical findings were such as to warrant a diagnosis of primary atypical pneumonia (28). These patients all had unequivocal serologic evidence of pneumonia; a total white blood count of below 15,000 and temperature re-

cordings of at least 102° F., before therapy was instituted. Several of these patients were exceptionally ill and none of those treated with penicillin had shown any improvement. Of the 11 patients from whom serum specimens were obtained at appropriate times there were seven who showed a cold agglutinin titer of 1:40 or more. Each of the two most severely ill patients had titers of 1:640, the remainder falling between 1:40 and 1:60.

In 14 of the 16 patients there was prompt initial symptomatic improvement and return of temperature to within normal range within 72 hours following institution of aureomycin. There were several whose response was extraordinarily rapid. The remaining two patients while showing clinical improvement remained febrile for somewhat longer periods. One patient appeared to relapse after drug had been stopped following good initial response. One of the patients who had shown a

good initial response appeared to relapse after the drug had been stopped, with return of low-grade fever and an increase in the X-ray evidence of pneumonia. This sequence of events has been observed to occur in other patients treated with aureomycin in another study (29). There follows a detailed case history of a patient with primary atypical pneumonia in whom treatment was carried out by the intravenous route alone. This case is summarized in Figure 4.

Case 5. T. B., white, male, age 17 (UCH 163710). This patient entered the University of California Hospital on February 25, 1949, complaining of a dry, hacking cough of two weeks' duration. On the day preceding hospital admission he had developed substernal pain and fever, and cough had become productive of a moderate amount of yellowish sputum. He had had asthma as a child but had suffered no attacks during the past 10 years.

On examination the temperature was 38.5° C., pulse 100 per minute, and respiration 20 per minute. The patient did not appear acutely ill but coughed frequently.

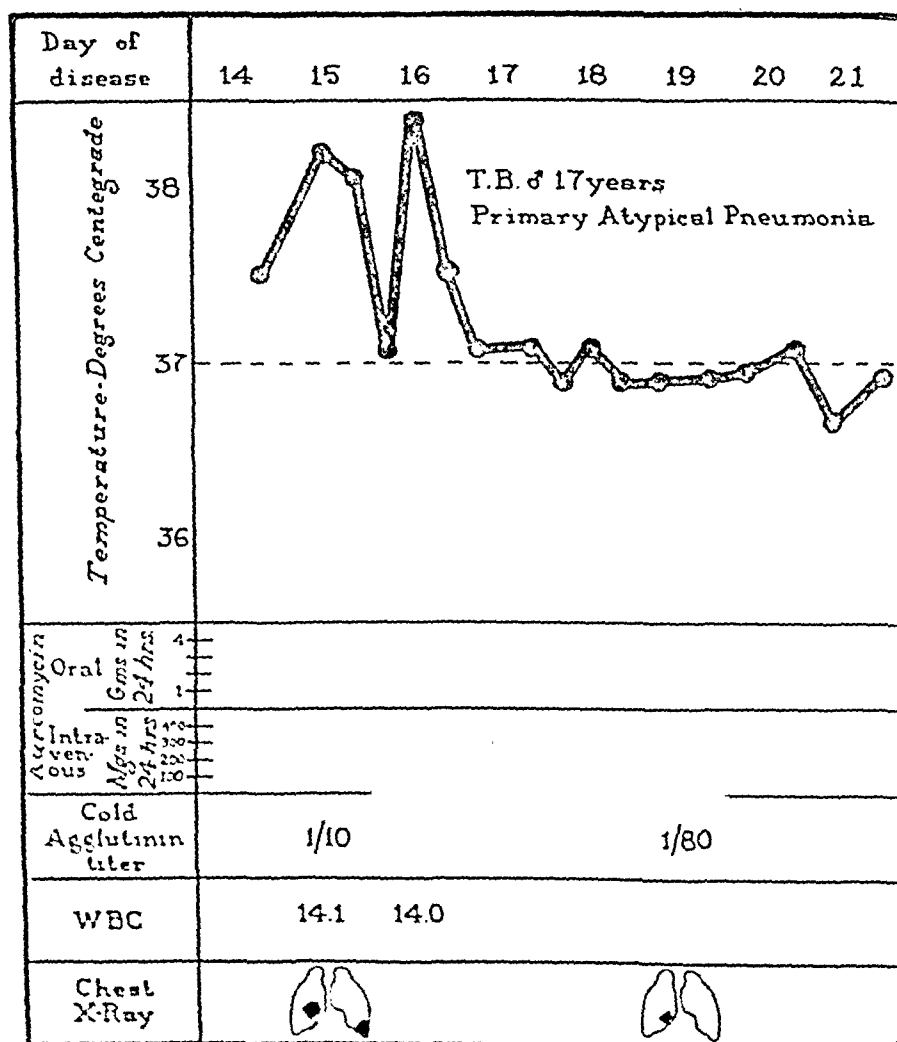


FIG. 4

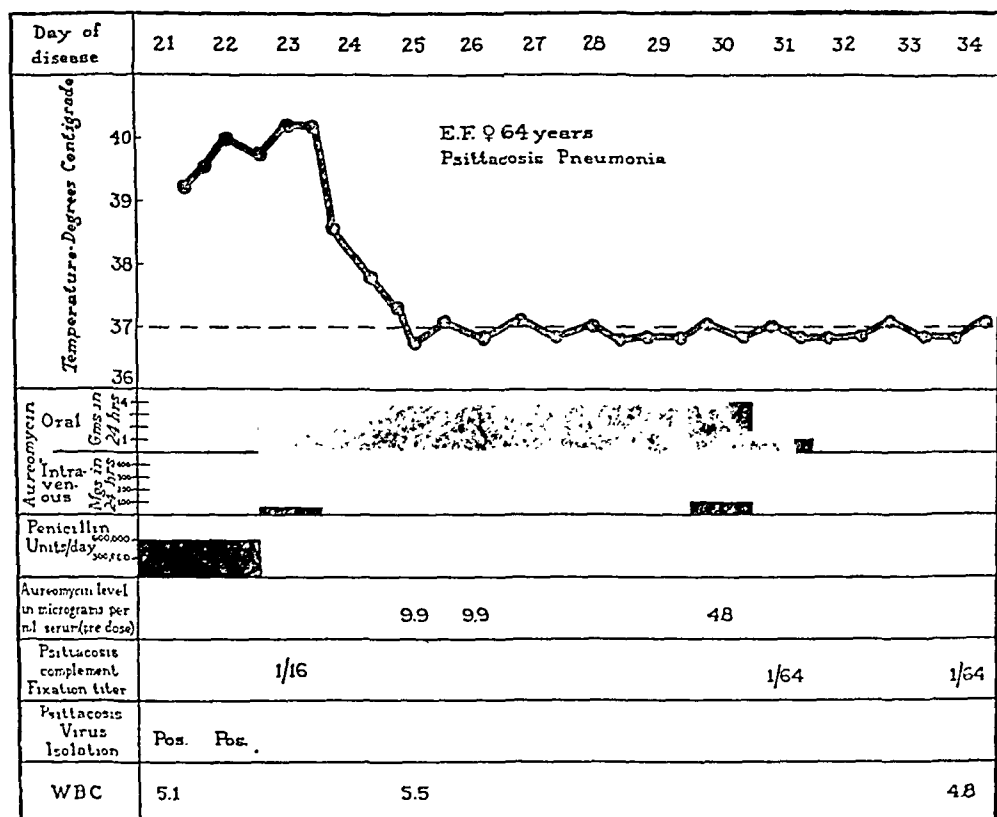


FIG. 5

Squeaking fine and coarse rales were heard in both lung bases posteriorly. Physical examination was otherwise within normal limits.

Urinalysis was normal. Complete blood count showed: Hemoglobin 16.5 grams per cent; red blood cells 5,100,000 per cubic mm.; white blood cells 14,150 per cubic mm.; neutrophils 77 per cent; lymphocytes 12 per cent; monocytes 11 per cent. Corrected sedimentation rate (Wintrobe) was 17 mm. per hour. Throat culture was negative for pathogens. Chest X-ray revealed pneumonitis in both right and left lower lobes. Cold agglutinins were present in a dilution of 1:20 at the time of entry. This titer rose to 1:80 five days later.

100 mg. of aureomycin in leucine diluent was administered intravenously every eight hours from February 26 to March 1. The patient became afebrile and improved symptomatically within 24 hours. The rales disappeared from the chest by February 27. A chest film on March 2 revealed almost complete clearing of the previously noted pneumonitis.

Psittacosis

Trial of aureomycin in psittacosis was of particular interest because laboratory investigation suggested that this drug was highly effective *in vivo* (7). Penicillin has been found to be an ef-

fective therapeutic agent in a small number of reported cases as reviewed by Goggio (30). A larger number of cases with favorable therapeutic response is unreported (31).

Three patients with a diagnosis of psittacosis have been treated with aureomycin. In one the diagnosis was established by isolation of the virus and demonstration of an increase in complement-fixing antibody titer. The second diagnosis was based on the presence in the convalescent serum of complement-fixing antibody in a titer of 1:256. In the third, the diagnosis was based on a history of contact with infected birds, typical clinical course, and the fact that the patient's wife, who was ill at the same time, was shown to have a complement-fixing titer of 1:64. This patient himself did not develop complement-fixing antibody in significant titer. The diagnosis in the last two patients must be considered incompletely established. All three of these patients appeared to respond to aureomycin therapy.

The first case, E. F., is presented in detail below (Figure 5).

Case 6. E. F., white, female, age 64 (SFH 25740). The patient entered the San Francisco Hospital December 12, 1948, giving a history of anorexia of four weeks' duration followed by generalized aching and malaise for the three weeks preceding hospital entry. Two weeks before admission she developed a severe productive cough and high fever accompanied by sweats. On October 26, 1948, she had been given a parakeet which died one week later. No other members of the household had been ill, but two cases of psittacosis had recently been diagnosed as arising from parakeets obtained from the same source.

Past history was irrelevant except that the patient had had chronic productive cough for 40 years.

On examination the temperature was 39.5° C., pulse 86 per minute, and respiration 32 per minute. The patient appeared severely ill and drowsy. Dullness, diminished breath and voice sounds and fine and medium rales were noted over both lower lung lobes. Physical examination was otherwise essentially negative.

Urinalysis was normal. The blood count showed: Hemoglobin 11.2 grams per cent; red blood cells 3,210,000 per cubic mm.; white blood cells 5,150 per cubic mm.; polymorphonuclear leukocytes 60 per cent; eosinophiles 2 per cent; lymphocytes 38 per cent. Corrected sedimentation rate (Wintrobe) 42 mm. per hour. Mazzini test was negative. Blood culture was sterile. Throat culture revealed no pathogens. Stool culture was negative. Sputum was repeatedly negative for tubercle bacilli. Cold agglutinins were not demonstrated on December 16 and December 22. Complement fixation reaction for psittacosis was positive in a titer of 1:12 on December 14 and rose to 1:64 within a week. Psittacosis virus was recovered from the sputum by inoculation of cotton rats and mice. Agglutinations for typhoid, paratyphoid, and brucellosis were negative December 13 and 14. Complement-fixation for Q fever was negative. Chest X-ray revealed patchy accentuation of pulmonary markings radiating from both hila which was believed compatible with bronchopneumonia.

The patient received 300,000 units of penicillin procaine complex twice daily on December 13 and 14 at which time virus was still demonstrable in the sputum and fever unabated. On the evening of December 13 the patient was started on aureomycin 1 gram by mouth every six hours which she received until December 22.

50 mg. was administered intravenously at the time of the first oral dose. The patient became afebrile within 24 hours and remained so subsequently. Symptomatic improvement was prompt although the patient suffered from nausea and occasional vomiting considered due to aureomycin which was severe enough on one occasion to necessitate an intravenous dose of 100 mg. On December 17 the chest was negative to physical examination. Chest film taken on December 18 showed marked diminution of the inflammatory reaction previously seen. The patient was discharged as well on December 27, 1948.

The second patient, a male aged 38 years, was treated with aureomycin on the 12th day of the

disease and, following failure of response to repository penicillin, showed prompt symptomatic improvement. The temperature, which had been as high as 105° F. before therapy, dropped rapidly but a low-grade fever persisted for eight days.

The third patient, a male aged 47 years, who was admitted to the hospital on the fifth day of the disease, was treated with repository penicillin in doses of 300,000 units twice a day for five days. At the time penicillin was stopped the patient's temperature was 102° F. During the following three days, the patient's temperature fell gradually to normal but rose again to a level of 104° F. on the 15th day of the disease. Following institution of aureomycin therapy at this point, the temperature fell to below 100° F. within 48 hours and, with the exception of a single spike, remained below 100° F. thereafter with occasional temperature elevations to 99.4° F. until the 23rd day of illness. Within two days after starting the drug, marked symptomatic improvement was noted. These cases illustrate relatively poor response to repository penicillin and subsequent good response to aureomycin.

Miscellaneous Diseases

A small group of patients suffering from bacterial pneumonias and pneumonia of unknown etiology was treated with aureomycin. In addition, one patient with proven primary coccidioidal pneumonia received aureomycin in full dosage for five days without demonstrable effect. Of two patients with pneumococcal pneumonia, one became afebrile within 12 hours; the other continued to have low-grade fever after an initial critical response until the introduction of penicillin treatment. One child who had chronic cystic fibrosis of the pancreas on which staphylococcal pneumonitis was superimposed evidenced slight temporary depression of fever followed by relapse. In five patients with pneumonia of unproven etiology response to aureomycin varied from none to excellent. One patient died of septicemia and pneumonia due to *Pseudomonas aeruginosa* in spite of exceedingly high serum levels of aureomycin during one and one-half day's treatment.

Two patients with generalized peritonitis of appendiceal origin, but whose infecting organisms were unknown, responded satisfactorily to aureo-

mycin after becoming worse while under penicillin therapy. One patient with a peritoneal abscess, from which *E. coli* was cultivated, recovered promptly but the beneficial result was probably not attributable entirely to aureomycin, since colpotomy was done. One chronic fecal fistula following appendectomy did not close during two weeks treatment with aureomycin.

Dramatic healing was observed in an infant suffering from chronic pyoderma due to a penicillin-resistant staphylococcus. One of three patients with pemphigus appeared to go into remission while being treated with aureomycin, but new lesions developed 12 days after treatment was stopped. Another patient with pemphigus was treated during a period of apparent spontaneous improvement, making the result difficult to evaluate. A third patient did not improve. One patient who had herpes zoster ophthalmicus healed dramatically within one week of the beginning of aureomycin treatment. No apparent beneficial effect was observed in one patient who developed varicella while receiving aureomycin. Another patient with chickenpox developed no new lesions after aureomycin was begun on the second day of eruption. One patient who had erysipelas responded satisfactorily to aureomycin. The fever and bacteremia in two patients suffering from subacute bacterial endocarditis due to penicillin-resistant *Streptococcus fecalis* disappeared during treatment with aureomycin but recurred one and seven days respectively after the termination of long courses of therapy. In one of these patients the blood culture became sterile only after serum concentrations of aureomycin of 10 to 20 μ g. were attained.

Four patients with primary chancroidal ulcers of the penis and inguinal adenitis responded promptly to aureomycin therapy by rapid healing of the ulcers and subsidence of adenitis. One patient with inguinal adenitis due to lymphopathia venereum also appeared to respond satisfactorily.

No definite therapeutic effect was observed in three patients suffering from infectious mononucleosis. One patient with stomatitis, balanourethritis, conjunctivitis, and arthritis did not manifest therapeutic effect from aureomycin. No beneficial effect was observed from the treatment of Hodgkin's disease, acute leukemia, or carcinoma of the parotid gland.

DISCUSSION

Available data indicate that aureomycin will assume an important place in the physician's armamentarium. Its role as the preferred agent in the treatment of primary atypical pneumonia, psittacosis, leptospirosis, and infections of the urinary tract by *Streptococcus fecalis* seems likely to be established. The rarity of development of drug-resistant organisms would suggest its curative or suppressive value in the treatment of urinary tract infection due to susceptible organisms either in the presence or absence of obstructive lesions. Preliminary data would also indicate the promise of aureomycin in chancroid and lymphopathia venereum infections. Although acute brucellosis appears to be generally amenable to treatment with this antibiotic, its value in chronic brucellosis remains to be proven. In spite of excellent results in the majority of Q fever infections, treatment failures have been encountered. Results reported by others in typhus and Rocky Mountain spotted fever in small series of patients appear even more favorable (6, 10, 14, 16, 19, 22, 24). As yet inadequately explored is the place of aureomycin in the treatment of herpes zoster, varicella and herpes simplex.

As an alternative agent to penicillin in infections due to gram-positive cocci and Neisseriae, aureomycin offers great promise, especially where penicillin resistance is encountered. Furthermore, because of its very wide spectrum of activity, aureomycin may assume a dominant role in the treatment of acute infections where the exact etiology is unknown, as in "atypical" pneumonias of all sorts. The relative value of aureomycin and streptomycin in the treatment of infections due to gram-negative organisms susceptible to both agents cannot yet be estimated, but at least aureomycin will serve as an alternative agent in this field. Comparison of aureomycin and chloramphenicol in the treatment of rickettsial and certain viral and bacterial diseases must await carefully controlled studies.

In infections due to *E. typhosa* and the *Salmonellae*, aureomycin appears to be of limited usefulness. Although definite beneficial effect appears to result in some cases, frequent failures occur and cannot be entirely explained on the basis of inadequate serum concentrations. The activity of

aureomycin against infections due to *Ps. aeruginosa* and *Pr. vulgaris* appears to be very limited, even in the urinary tract where very high concentrations of the antibiotic are usually attainable (26).

Unlike penicillin and streptomycin, but somewhat reminiscent of the sulfonamide drugs, aureomycin often appears to exert a greater or lesser suppressive effect on the infecting agent without producing its complete destruction. Aureomycin may interfere with the development of complement-fixing antibodies in psittacosis and cold agglutinins in primary atypical pneumonia (29).

With the exception of nausea and vomiting, aureomycin compares favorably with penicillin in lack of significant toxic effects in relation to anti-infective activity. Even this side effect may be avoided by use of the intravenous route of administration. Furthermore, approximately one-tenth as much drug need be given intravenously as by mouth. Reduction of the oral dose or further purification of the antibiotic may also obviate gastrointestinal symptoms.

SUMMARY AND CONCLUSIONS

1. Aureomycin was used in the treatment of 116 patients with infections due to a variety of bacterial, rickettsial and viral agents.

2. The only significant toxic symptom encountered frequently was nausea and vomiting.

3. The intravenous route of administration, used in 68 individuals, proved to be an effective method of therapy, both when used to supplement oral medication to produce high serum concentrations of aureomycin, or to replace the oral route in the control of nausea and vomiting.

4. Aureomycin appeared to exert beneficial effect in acute brucellosis, leptospirosis, Q fever, primary atypical pneumonia, psittacosis, chancroid, lymphopathia venereum, generalized peritonitis, and many infections of the urinary tract.

5. Beneficial effects from aureomycin therapy appeared to be limited or absent in typhoid, *Salmonella*, *Shigella* infections, coccidioid pneumonia, infectious mononucleosis, varicella and subacute bacterial endocarditis due to *Streptococcus fecalis*.

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FURTHER EXPERIENCES WITH INTRAVENOUS AUREOMYCIN THERAPY. A STUDY OF 116 CASES^{1,2}

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The need for developing routes of administration for aureomycin other than oral has been apparent since the initial clinical trials with the antibiotic in the early months of 1948. To fill this need, studies on intravenous aureomycin were carried out simultaneously in the summer and fall of 1948 at St. Vincent's Hospital in New York by Rottino, Sanders, and Philip (1) and at the Harlem Hospital by Hill, Wright, Prigot, and Logan (2). The present report is an extension of the earlier work.

In the study at St. Vincent's, patients suffering from Hodgkin's disease received large doses of aureomycin intravenously up to 4 grams daily and toxicity data were thus obtained since the dosage was pressed until nausea and vomiting occurred. This knowledge has permitted us to proceed conservatively and without ill effects in using the intravenous route for therapy in numerous clinical entities. Detailed results on the clinical studies will be published elsewhere (3). At this time, it may be said that the intravenous method of administration has frequently produced dramatic therapeutic response and has proved to be an entirely practical procedure producing, at therapeutic levels, little or no side reactions.

It is to be noted that kidney and liver function tests made both at St. Vincent's and Harlem Hospitals at no time showed variation from the normal, even after massive doses of aureomycin intravenously were maintained for one week.

Indications for intravenous therapy

In general, the occasions are fairly obvious when the intravenous administration of aureomycin is

to be preferred to the oral or intramuscular routes. The desirability of rapidly obtaining a high aureomycin blood level with minimum discomfort is clear where:

- a. fulminating disease is present and the amount of antibiotic required would almost certainly cause gastro-intestinal disturbances;
- b. the patient does not have the benefit of gastro-intestinal absorption because he is vomiting (due to peritonitis, intestinal disturbances, etc.) or because he is in a comatose or semi-comatose condition; and
- c. the patient has attempted oral medication and has experienced marked discomfort without getting relief from the condition which was the original indication for therapy.

In addition to the extreme circumstances where intravenous aureomycin is urgently necessary, the most common use to which the intravenous method has been put by us is as an adjuvant form of therapy. From the previous blood level studies carried out at St. Vincent's, it was known that aureomycin injected into the blood reached a high level quickly and was also excreted with moderate rapidity (see Figure 1). This fact, as well as our reluctance to use intravenous medication unnecessarily, stimulated the study of combined intravenous and oral medication. It is in this realm that we believe that intravenous administration of aureomycin will find its greatest usefulness.

MATERIALS AND METHODS

Aureomycin hydrochloride³ is a yellow, highly soluble powder available in 50-mgm. vials. The procedures for preparing and administering the antibiotic followed in this study have been essentially the same as those recommended by Rottino, Sanders, and Philip. Unless special diluents are being investigated, the contents of each 50-mgm. vial are thoroughly dissolved in about 2 mls. of distilled water. If it is necessary or desirable to give

³ We wish to thank the officials of the Lederle Laboratories who so generously supplied large amounts of aureomycin and thus made the present study possible.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

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the patient parenteral fluids, the infusion indicated is started. The contents of the desired number of 50-mgm. vials of aureomycin in distilled water are then pooled and diluted in proper amounts of 1/6 M. lactate (Hartman's or Lactate-Ringer's) solution. After thorough mixing, the aureomycin in lactate is given as a rapid infusion. The aureomycin in distilled water is not mixed with the sodium lactate until the infusion is assembled because Harned and his associates (4) found that the antibiotic is stable in an acid medium and loses potency in an alkaline environment.

As far as the amount of lactate for the infusion is concerned, it is our impression that only an approximate quantity is necessary since the lactate merely serves as a vehicle for the antibiotic. It is important to remember that the buffering action of the lactate is helpful since aureomycin hydrochloride in distilled water has an approximate pH of 3.5 whereas the pH of the final mixture in lactate is approximately 5.5. The following quantities of 1/6 M. lactate solution as infusion vehicles for aureomycin varying from 50 to 1,000 mgms. are suggested. The schedule in Table I is on an entirely empirical basis and is one which has been satisfactory in our hands:⁴

TABLE I

A suggested ratio of aureomycin HCl to 1/6 M. sodium lactate for intravenous infusions

Aureomycin mgms.	to	1/6 M. sodium lactate mls.
1000		500
350-500		250
200-350		150
200		100
100		50
50		20

The bio-assay method

The bio-assay test for determining the amount of aureomycin in various body fluids was originated at the Lederle Laboratories by Dornbush and Pelcak (5). Since any attempt to study methods of administration depends largely upon an exact adherence to the bio-assay method, care was taken to make certain that the technique used in this study was in complete agreement with the test at Dornbush's laboratory.⁵

Comparative data: intravenous plus oral vs. oral medication alone

In presenting Figures 1-4 an attempt has been made to cull our experience with 116 patients on intravenous aureomycin therapy so that a basis

may be provided for therapeutic manipulation which may be useful in various clinical problems. During recent months, patients received various schedules of aureomycin therapy ranging from a single intravenous injection of 20 mgms. in an infant to a combined intravenous-oral course of an antibiotic in an adult, in whom the intravenous portion alone consisted of 76 500-mgm. infusions for a total of 38 grams (34 days of 500 mgms. b.i.d. and 42 days u.i.d.). In addition to the intravenous medication, 47 grams of adjuvant oral therapy were given. This patient, V. L., is alive and well and is apparently recovering from a chronic disease.

The primary purpose in demonstrating the results of the bio-assay in the following charts is to show the physician how he may proceed in a general fashion in obtaining aureomycin blood levels to satisfy special clinical requirements.

Figure 1 emphasizes the high concentration of aureomycin which can be obtained in the blood following a single injection of 1 gram intravenously. With two hours, a peak of 20 μ g. was reached but there was a fairly rapid drop to 10 μ g. two hours later. Although these high concentrations were not maintained for a long period of time under the given situation, a level of 5 μ g. was present up to 14 hours following the injection. This, of course, is a significant level which would be more than sufficient to combat most infections. Even after 24 hours, a significant level of 2 μ g. resulted. The technique of injection of 1 gram at a time appears to have little hazard judging the ease with which ten patients received such injections.

The advantage of using oral therapy in combination with intravenous therapy is illustrated in Figure 2. When compared with a single intravenous injection of 1 gram, the smaller intravenous injection of 350 mgms. given in conjunction with 250 mgms. by mouth every four hours produced significant blood levels which fluctuated between 3.2 and 6.4 μ g.

If this comfortable oral and intravenous schedule is continued for 24 hours (Figure 3), we see a dramatic rise to 12.8 μ g. with an excellent maintenance of aureomycin concentration for as long as 12 hours after cessation of therapy. Figure 4 also illustrates the difference between the oral regime

⁴ Additional work with intravenous aureomycin since the presentation of this paper has made it apparent that the amounts of 1/6 M. sodium lactate can be reduced to half those shown here.

⁵ We wish to thank Messrs. Dornbush and Pelcak for their cooperation and for providing purified antibiotic to be used as standard test material.

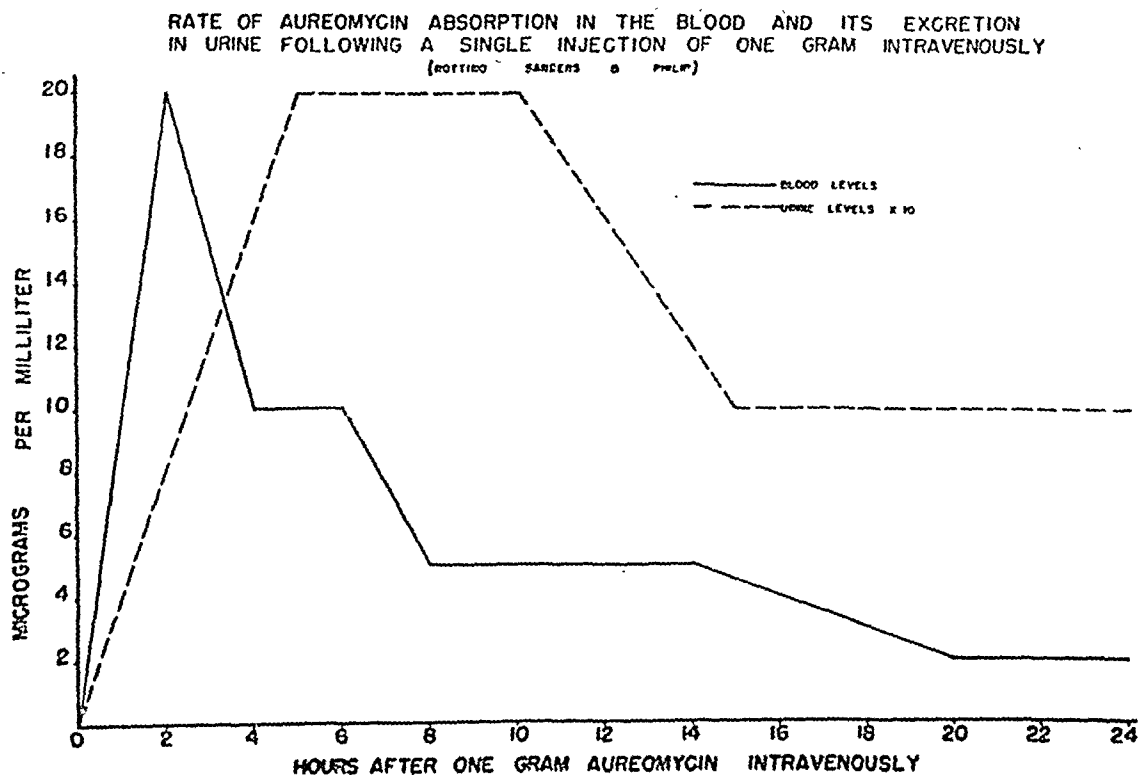


FIG. 1

men of 250 mgms. every four hours and the combined oral-intravenous schedule. On the oral therapy there is a slow rise with the level not exceeding 2 μ g. for the first ten hours. It is only

within 12 hours after the first capsule is taken that a concentration of 4 μ g. per ml. is reached in the blood. The peak of 5 μ g. is reached and maintained from 18 to 34 hours.

COMPARISON BETWEEN COMBINED INTRAVENOUS-ORAL AUREOMYCIN ADMINISTRATION AND A
SINGLE INJECTION OF ONE GRAM OF AUREOMYCIN INTRAVENOUSLY

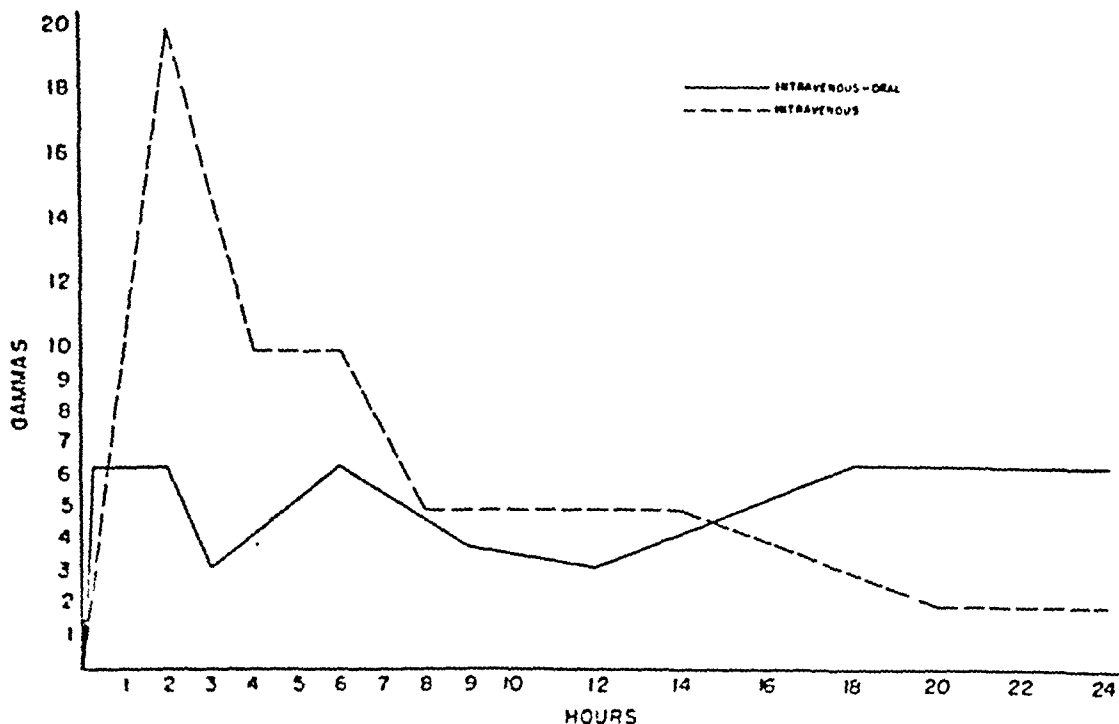


FIG. 2

COMPARATIVE BLOOD LEVELS
FOLLOWING ORAL AND INTRAVENOUS-ORAL ADMINISTRATION OF AUREOMYCIN

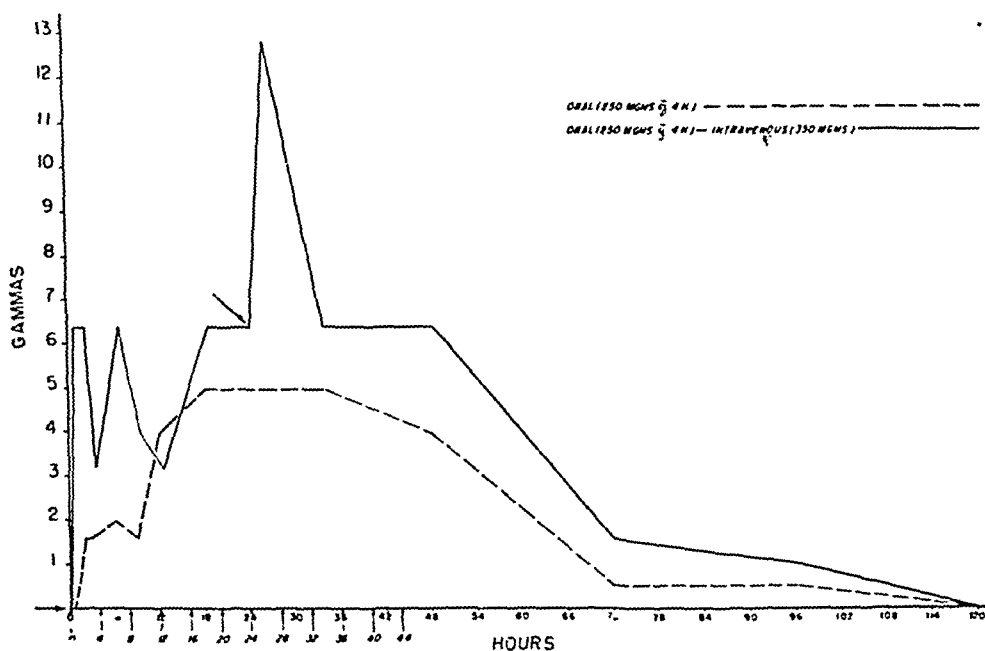


FIG. 3

By utilizing the information in Figures 2 and 3, it should be possible to maintain aureomycin concentrations at any desired level, subject, of course, to individual variations. The factor of individual

variation to similar dosage schedule has been observed with penicillin and streptomycin therapy. That the same factor must be considered in aureomycin therapy is apparent from Figure 4. The

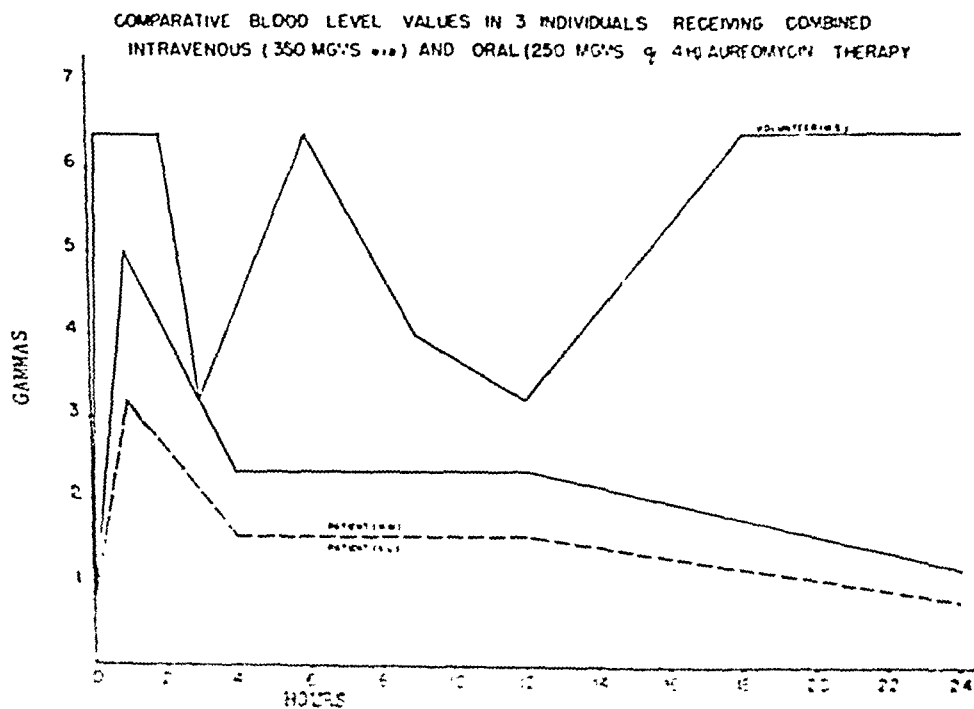


FIG. 4

volunteer, M. S., was not a demonstrably ill individual and reached a peak concentration of 6.4 $\mu\text{g.}$ in his blood within 20 minutes after the intravenous injection of 350 mgms. This level fluctuated during the next 24 hours between 3.2 and 6.4 $\mu\text{g.}$ In contrast, patient H. M., suffering from infectious mononucleosis, and patient V. L., a post-hepatic cirrhotic, who were on the same intravenous and oral schedule as the volunteer, reached a peak of 3.2 $\mu\text{g.}$ and a maintenance level of between 1 and 2 $\mu\text{g.}$

Enhanced aureomycin blood levels by the use of caronamide

Since it has been shown that the blood concentration of penicillin can be enhanced by the oral administration of caronamide (6), an attempt was made to ascertain the effects of this drug (4-Carboxyphenylmethansulfonamide) on aureomycin absorption. To date, three⁶ patients have been studied and the result suggests that caronamide raises the blood level of aureomycin.

⁶ A temporary shortage of parenteral aureomycin has limited the number of patients under study with caronamide.

This phase of the work has been of particular interest to us since the assumption has been made by Beyer (7) that the action of caronamide in relation to penicillin absorption depends on the blockade by caronamide of specific enzyme systems responsible for penicillin transport through the renal tubules. Strauss, Richburg, Saba, and Alexander (6) have stated that caronamide has no effect on the plasma concentration of streptomycin when the antibiotic is given in multiple intramuscular injections. Inasmuch as aureomycin and streptomycin are derived from related microbial sources, it is curious that an enhancement action has been demonstrated.

One of the first patients to receive caronamide, 4 grams by mouth every four hours, was H. M. As is apparent from Figure 5, the increase of the aureomycin level following the use of caronamide was not dramatic, since a peak of 4 $\mu\text{g.}$ was reached in association with caronamide as against a peak of 3.2 $\mu\text{g.}$ when aureomycin was used alone. It may be significant that in the last 12 hours, a level of 2 $\mu\text{g.}$ was maintained when caronamide was given with the aureomycin, whereas without this adjunct the aureomycin level dropped to less than 1 $\mu\text{g.}$

EFFECT OF ORAL ADMINISTRATION OF CARONAMIDE ON ENHANCING AUREOMYCIN BLOOD LEVEL 350 MILLIGRAMS AUREOMYCIN IV q.i.d. PLUS 250 MILLIGRAMS $\frac{1}{2}$ 4 HRS ORALLY. CARONAMIDE 4 GRAMS EVERY 4 HOURS

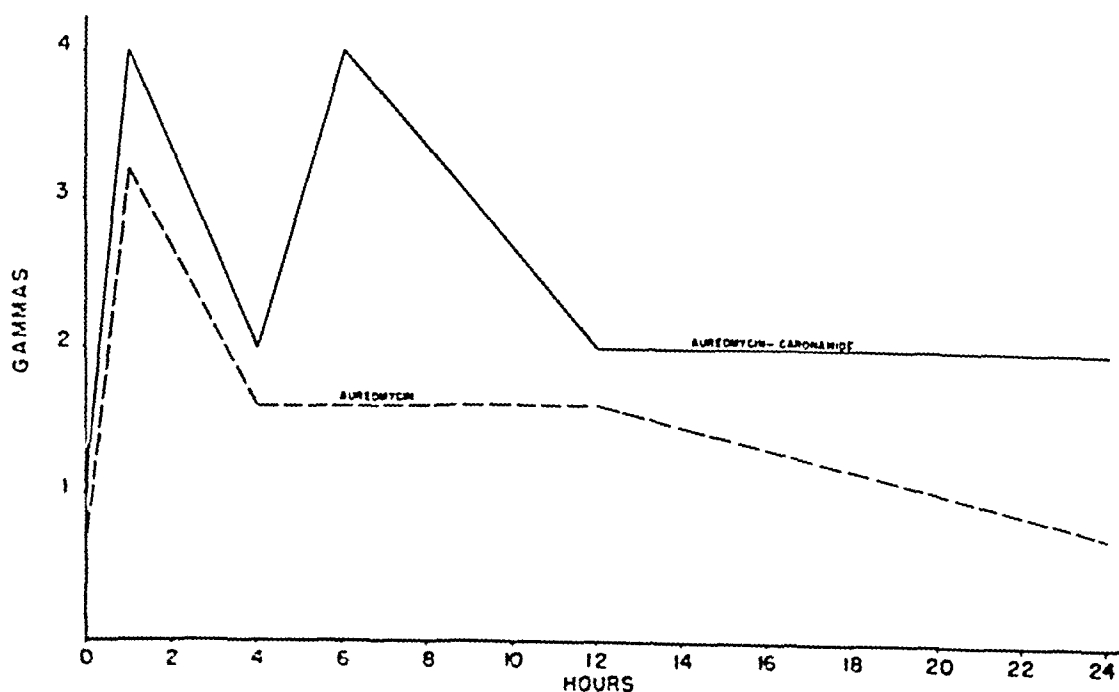


FIG. 5

EFFECT OF ORAL ADMINISTRATION OF CARONAMIDE ON EXCRETION OF AUREOMYCIN

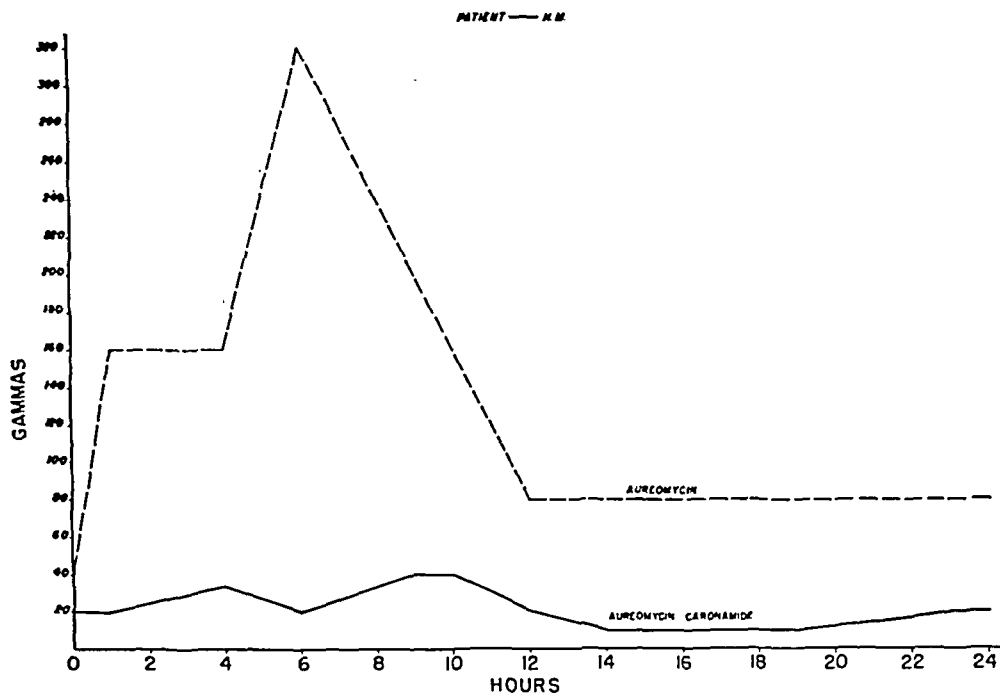


FIG. 6

TABLE II
Phlebitis following intravenous aureomycin therapy

Group	Number of patients	Number of injections	Mgms. per injection	Phlebitis	No Phlebitis
I	60	6-15	200 to 250	28 (mild)	32
II	35	1-5	250 to 350	0	35
III	21	15 to 76	250 to 500	21	0

tions have been made into the vein. Secondly, the amount of aureomycin which is injected into the vein appears to have some bearing on the formation of phlebitis.

The 116 patients who received aureomycin intravenously reacted in the following fashion (see Table II).

Although many of the patients who received the antibiotic intravenously showed a phlebitis, in the majority of instances the tenderness, induration and rigidity of the vessel disappeared within periods ranging from a few days to several weeks.

The whole problem of phlebitis was to some extent minimized when care was taken to attach intravenous infusions only after sharp needles were well into the veins. Another helpful point of technique was the practice of following the simple

fundamentals of intravenous therapy; *i.e.*, giving the first infusions in distal veins, later injections proximally, and varying the sites of injection by using alternate hands and arms. As a matter of fact, phlebitis has never been sufficiently serious to prevent a patient from receiving several injections. It is only the hypersusceptible individual who is unable to receive many injections. Occasionally, when numerous infusions are required, the presence of phlebitis makes continued therapy difficult or impossible.

One other complication was noted in three of the 116 patients. In one instance, the patient experienced a chill resulting in a fever of 101° within one hour after the intravenous administration of aureomycin. The chill was moderately severe and the fever lasted approximately one hour. Repetition of the infusion the next day produced essentially the same reaction in this patient and oral medication was instituted in place of the infusions. In a second patient, a similar reaction occurred, the fever going to 105° ; when intravenous medication was cautiously continued in this instance, however, no further reaction was observed and the patient was able to take a course of 15 injections of 500 mgms. each without fur-

EFFECT OF ORAL ADMINISTRATION OF CARONAMIDE ON ENHANCING AUREOMYCIN BLOOD LEVEL.
350 MGMS AUREOMYCIN IV, *q.i.d.* PLUS 250 MGMS $\frac{1}{2}$ 4 H ORALLY CARONAMID 4 GRAMS $\frac{1}{2}$ 4 H

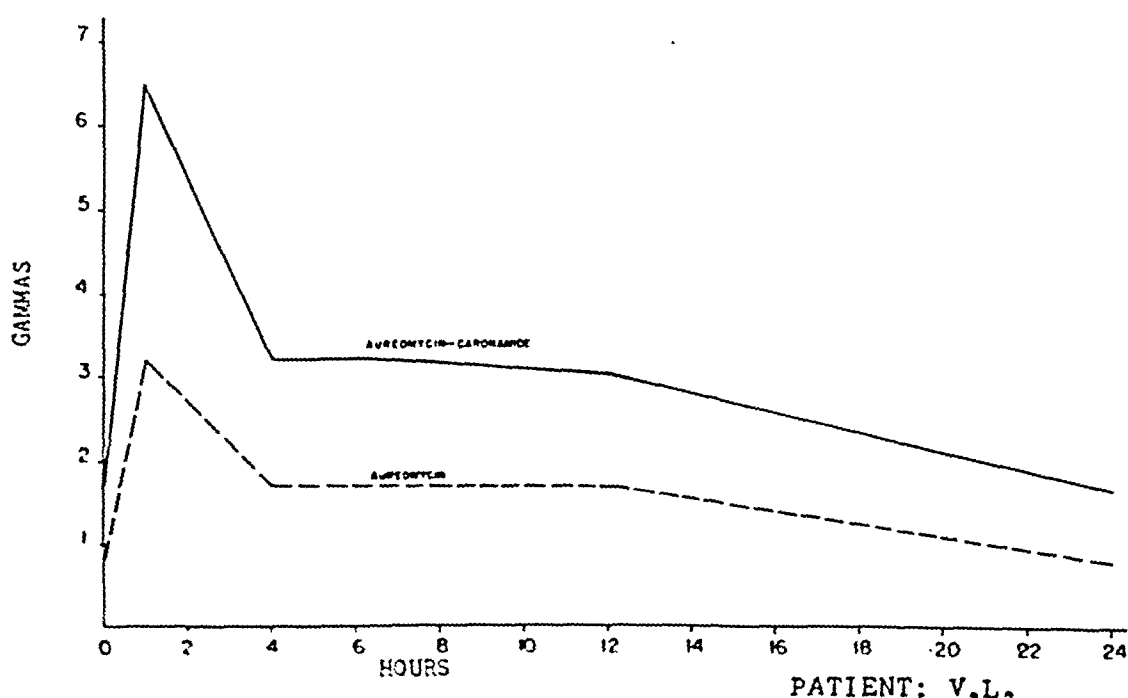


FIG. 7

ther untoward reaction. In a third patient, who had a fever of 103° before the infusion was administered, there occurred a chill and rise in temperature to 105° four hours after the infusion. A repetition of the process on the following day made us reluctant to use further intravenous therapy, particularly since the patient was able to take aureomycin by mouth.

Aureomycin has shown little capacity to produce sensitization. Nevertheless, it must be borne in mind that it is only a matter of time before the patient is found who will show allergic manifestations to this antibiotic. While no serious reactions were encountered in our series of 116 patients, it has been noted that cessation of therapy was necessary in two instances where chills and fever followed the antibiotic infusions.

Since reactions have been so infrequent and of a relatively mild nature, we believe further investigation is warranted regarding the advisability of using an initial test dose of 50 mgms. or less in each patient, before an active therapeutic schedule is begun by vein.

CONCLUSIONS

1. The technique for administering aureomycin intravenously and indications for its use are discussed.
2. Blood concentrations and urinary excretions under several intravenous-oral and oral dosage schedules are submitted.
3. Absorption of aureomycin in the blood appears to vary with the individual.
4. It is suggested that caronamide may enhance blood levels of aureomycin and that it definitely decreases excretion of the antibiotic through the kidneys.
5. No serious toxic effects have been noted following intravenous aureomycin therapy. Only two of the 116 patients experienced febrile reactions. These reactions appeared to be of an individual nature since the same production lots failed to evoke reactions in other patients.

6. With the preparation of aureomycin now available phlebitis occurred in 30 to 40 per cent of the patients when the antibiotic was given intravenously.

7. Nausea and vomiting were not encountered as direct concomitants of intravenous therapy in this study. Gastro-intestinal symptoms were not noted even in a patient who had received 76 500-mgm. injections by vein.

8. We are of the opinion that intravenous aureomycin is a necessary mode of administration in some instances and a desirable one in others.

ACKNOWLEDGMENTS

We wish to express our appreciation to the attending and resident staffs of the following hospitals for their efforts in providing material and for assisting with the observations: V. A. Hospital, Coral Gables, Fla.; Jackson Memorial Hospital, Miami, Fla.; St. Francis Hospital, Miami Beach, Fla.; Alton Road Hospital, Miami Beach, Fla.; French Hospital, New York City; and St. Vincent's Hospital, New York City.

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PURITY STUDIES ON POLYPEPTIDE ANTIBIOTICS: BACITRACIN¹

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As part of a broader study on Counter-current Distribution, the question of the purity of available polypeptide preparations has been taken up. We have found it possible to fractionate gramicidin, tyrocidine, gramicidin-S, and bacitracin samples into several different polypeptide components. In each case, except with the tyrocidine fractions which have not been studied antibiotically as yet, a variation in activity with the different components has been noted. They differ also in the amino acids which they contain as well as in the proportion of these amino acids.

Bacitracin for the most part has been furnished by the Commercial Solvents Co. We are indebted to them and to Miss Johnson and Dr. Meleney for the bioassays. Bacitracin has not proved to be the most ideal type of substance to distribute because of its tendency to give skewed curves and because of its lack of stability. In attempts to distribute it in neutral 2-butanol/water, the curves obtained were typical transformation curves. 2-Butanol/water acidified with acetic acid was much more satisfactory and showed only slight transformation during a run. A typical result is shown in Figure 1. This was made with a sample of 46 unit material. A calculated curve superimposed on the weight curve showed the latter to be somewhat skewed. An activity curve also was skewed but seemed to indicate a single active substance being present in the major component. A considerable band of low activity occurred to the right. Absorption spectrum measurement indicated mixtures in tubes 40 to 60.

An interesting feature of this distribution is that a maximum activity of 66 units was indicated. This was on a dry-weight basis when dried at 100° in vacuum. Material recovered from the main band by freeze-drying was a white, highly hygroscopic powder which had an activity slightly less than the maximum obtained from the curve.

That it had lost a certain amount of activity through denaturation on isolation was confirmed by redistribution. In this case a small percentage of material of low activity appeared on the left of the main band.

Hydrolysis in 6 N hydrochloric acid followed by paper chromatography gave spots corresponding to phenylalanine, leucine, isoleucine, cystine, valine, histidine, ornithine, lysine, and glutamic and aspartic acids. Strong evidence is thus furnished that the active principle is a polypeptide of considerable size.

At about this stage of our work certain toxic manifestations were encountered by others on the clinical side and it became desirable to have more careful chemical investigations in order to see if some closely related toxic substance could be removed by fractionation. This problem can be approached in two ways, namely, higher numbers of transfers or a change to a more specific system. We have investigated both approaches.

An all-glass apparatus of simple construction has been devised which is shown in Figure 2. This particular one contains 108 units but it would appear that this number could be extended several

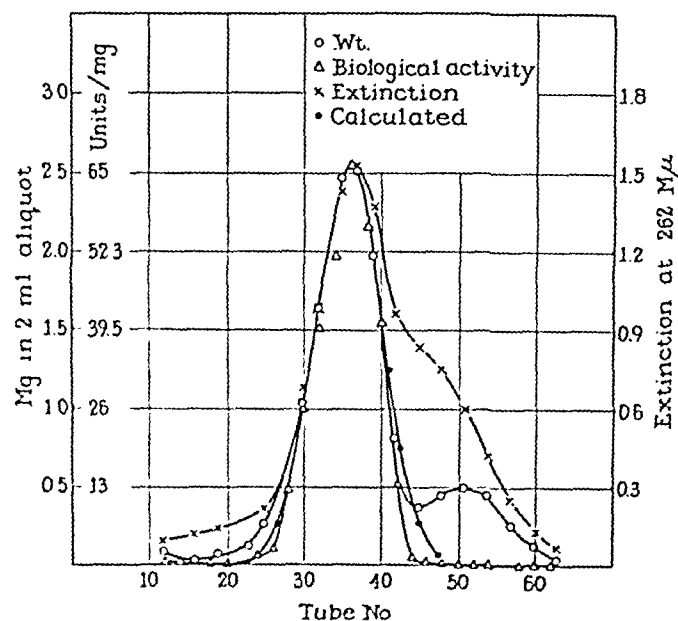


FIG. 1. 85 TRANSFER DISTRIBUTION OF BACITRACIN IN 2-BUTANOL/3% ACETIC ACID

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotic Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

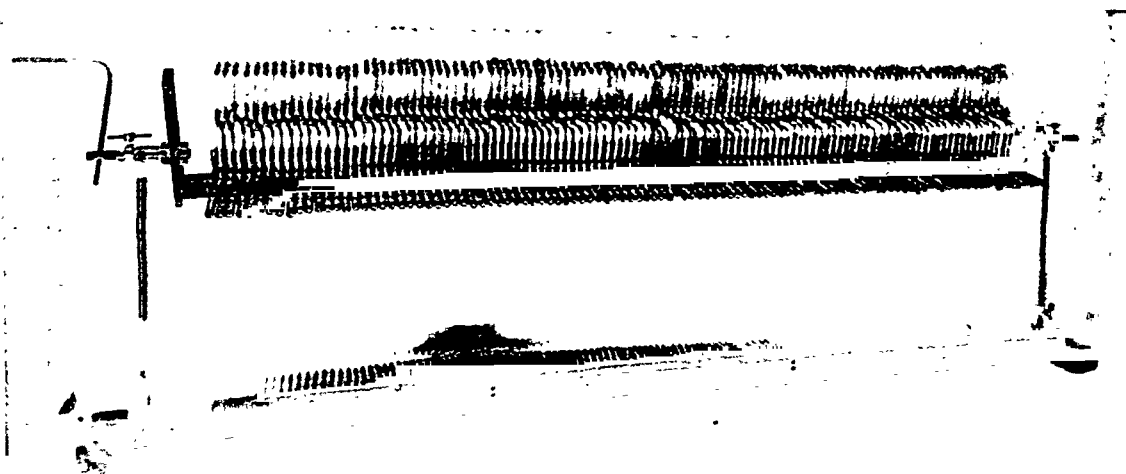


FIG. 2. GLASS COUNTER-CURRENT DISTRIBUTION APPARATUS CONTAINING 108 TUBES

fold if necessary for a specific problem. Enlarging is merely a matter of adding individual units. An interesting feature is that strong acids such as hydrochloric acid can be used.

A run on bacitracin using a system made with methanol, 0.1 N hydrochloric acid and chloroform gave the pattern shown in Figure 3. The overall aspects are not too different from those with the first system. Inactive material is shown at fractions 140 to 120 and a certain amount of foreign material is demonstrated on the left. Somewhat more inactivation was encountered during the run

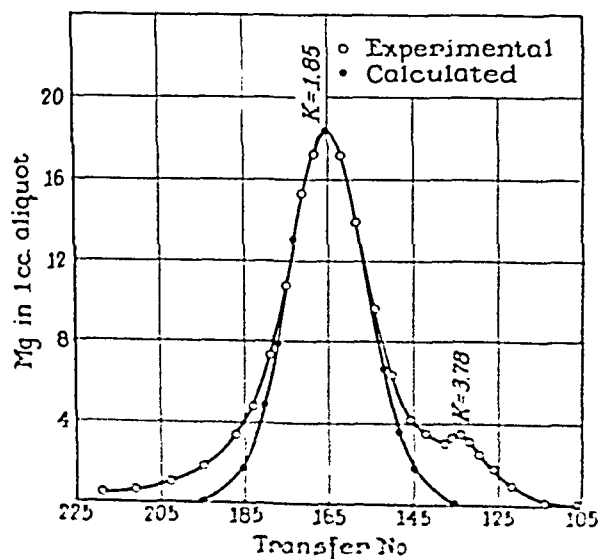


FIG. 3. DISTRIBUTION OF BACITRACIN IN A SYSTEM CONTAINING CHLOROFORM, METHANOL AND 0.1 N HYDROCHLORIC ACID

than with the acetic acid system. When inactivation in acid solution occurs, the transformation products appear on the left. When inactivation in neutral or alkaline solution occurs, the products appear on the right. A hydrochloride with rather nice properties was recovered from the peak tubes.

At least one sample of bacitracin was not entirely stable in the solid state. This sample origi-

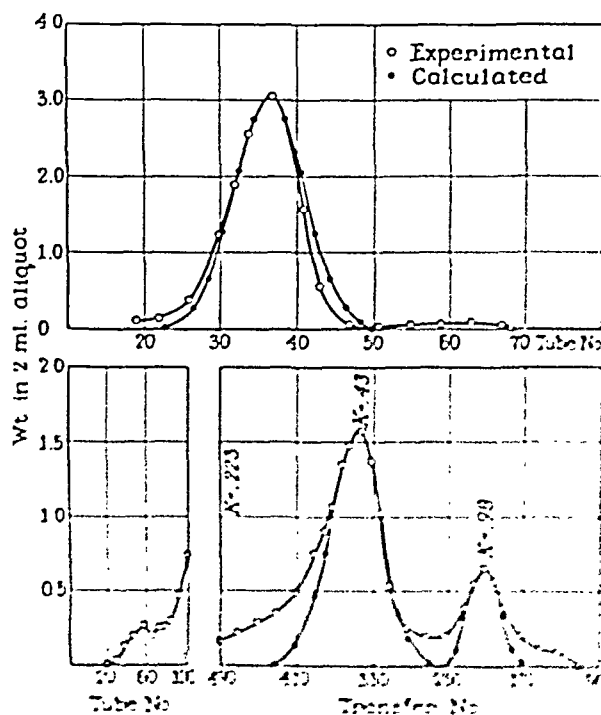


FIG. 4. DISTRIBUTION OF BACITRACIN IN A SYSTEM CONTAINING CHLOROFORM, METHANOL AND 0.1 N HYDROCHLORIC ACID

nally had an activity of 55 units and gave quite a good pattern, Figure 4, upper pattern, with 75 transfers and the acetic acid system. Only a small amount of inactive material on the right and left was obtained. After standing in the cold room for about nine months, the lower pattern was obtained with the same system but with the application of 490 transfers. The activity of the sample after nine months was 47 units. The fractions were recovered as usual by freeze-drying and, to our surprise and gratification, material recovered from the main band on one run had definite organization as shown in Figure 5. None of the fractions

recovered from other regions showed this organization. Furthermore, the recovered material had an activity of 60 units without further drying.

It would now appear fairly certain that this material is either a single substance or a mixture of very closely related substances. The evidence is not as rigorous as we would like because of inherent instability but at least the experience in two different systems points toward something definite on which to base future work.

Hydrolysis in boiling 6 N hydrochloric acid for 24 hours, evaporation of the excess hydrochloric acid, and distribution of the mixed hydrochlorides



FIG. 5. APPEARANCE OF PURIFIED BACITRACIN

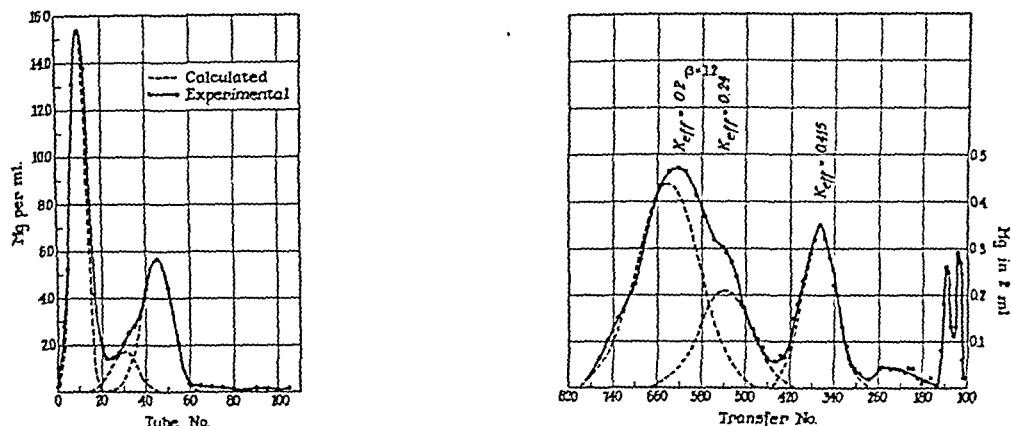


FIG. 6. DISTRIBUTION OF THE HYDROLYSIS PRODUCTS OF BACITRACIN

in a 2-butanol/ammonium acetate system has given the pattern shown in Figure 6. The bands from right to left are a dipeptide containing phenylalanine and isoleucine; peptide material containing phenylalanine, isoleucine, and apparently ornithine; phenylalanine; leucine; isoleucine; ammonium chloride; histidine; and, finally, a mixed band containing cystine, lysine, and aspartic and glutamic acids. Redistribution in other systems has permitted further resolution of the mixed band. Spotting of every tenth fraction on a broad paper chromatogram permits ready control of the separation.

The original "valine" spot has been isolated in crystalline form from tubes 60-70 but it is different from valine. It appears to be absent from the most highly purified material. All the spots indicated by paper chromatography have been isolated in crystalline form with the correct carbon and hydrogen analysis except lysine and ornithine. In addition, peptides have been isolated. The amino acids isolated were of the *L*-, *D,L*- and *D*-configurations, as follows: *L*-histidine, partially racemic *L*-leucine, *L*-cystine and *L*-glutamic acid, *D,L*-phenylalanine, *D,L*-aspartic acid, and partially racemic *D*-isoleucine.

A COMPARATIVE STUDY OF THE RENAL DAMAGE PRODUCED IN MICE BY VARIOUS LOTS OF BACITRACIN¹

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In recent publications it has been suggested that bacitracin grown by the deep-tank method shows a higher degree of nephrotoxicity than the bacitracin produced by surface culture methods (1). The studies being reported were designed to compare the two types of material from a strictly objective standpoint.

EXPERIMENTAL STUDIES

Various lots of bacitracin were selected for this study on the basis of their previous clinical use and their availability. Unfortunately, many of the lots could not be employed because of confusing clinical data resulting from variable and uncertain dosage schedules, the use of several lots in the same patient and the known presence of impaired renal function in some cases prior to the institution of bacitracin therapy.

Of the surface culture type of bacitracin lots BV-112, BV-140, BV-142 and BV-144 were used. BV-112 was selected because it was one of the first production lots and the other three BV lots had all been employed clinically and were said to show little evidence of nephrotoxicity. Five lots, B-471212, B-480210, B-480512, B-480420, and B-481102, produced by deep-tank fermentation were similarly chosen for study. Lot B-471212 was one of the earliest lots produced by the deep-tank method which was employed clinically. Lots B-480210 and B-480512 were included in the study because of clinical evidence of nephrotoxicity, while B-480420 by contrast was stated to have extremely little evidence of toxicity when employed clinically. Lot B-481102 was especially prepared by the deep-tank method; a synthetic medium was used containing glutamic acid, similar to the medium described by Meleney and his associates in which the original production of bacitracin was developed by the surface culture technic.

The bacitracin was administered intravenously to mice of the same strain in accordance with the specifications set up by the FDA (October 14, 1948). These specifications require that mice weighing between 18 and 25 grams be injected intravenously with a constant volume (0.5 ml. per 20-gram mouse) within a five-second period. The following doses were injected: 50, 100, 200, 300, 400,

and 500 units per 20-gram mouse. The animals were observed for a 72-hour period. The mice receiving the three lowest doses, namely, 50, 100, and 200 units per 20-gram mouse, were under constant observation during the 72-hour period in order to obtain fresh autopsy material in the event of death. The kidneys were removed immediately after death and placed in 10% formalin. The survivors were sacrificed at the end of the experimental period and their kidneys fixed in 10% formalin. A series of 10 mice injected with saline served as the controls.

RESULTS

Evidence of gross pathology as expressed by the degree of ischemia present was noted and recorded on a four-plus graded scale at the time of autopsy. Histologic sections were made of the kidneys. These sections were stained with hematoxylin-eosin and were graded for evidence of renal changes independently (LWS) without knowledge of the gross renal classification. Very little, if any, correlation between the degree of renal ischemia grossly and the extent of renal damage microscopically was found.

In Table I the acute toxicity of the various lots of bacitracin is shown along with the biologic activity of each lot.

In Table II the incidence of microscopic renal injury in mice injected intravenously with various lots of bacitracin in dosages of 50, 100, and 200

TABLE I
The acute toxicity of various lots of bacitracin when administered intravenously to mice and the biological activity of each lot

Lot No.	LD50	Potency units/mg.
BV 112	180±24	37
CSC B481102*	205±23	35
BV 140	210±17	30
BV 144	265±34	30
CSC B480512	305±35	51
CSC B480210	307±26	37
CSC B480420	325±28	52
CSC B471212	330±25	50
BV 142	333±50	31

* Prepared on synthetic medium
BV = Ben Venue lots
CSC = Commercial Solvents lots

¹ Presented at the Second National Symposium on Recent Advances in Antifolates Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antifolates Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

TABLE II

The incidence of microscopic renal injury in mice treated intravenously with various lots of bacitracin

Dose units/20 gm. Mouse.	BV112	BV140	BV142	BV144	CSC B480420	CSC B480210	CSC B480512	CSC B471212	CSC B481102*
50	2/6	3/6	3/6	2/6	4/6	4/6	2/6	4/6	5/6
100	5/6	6/6	4/6	3/6	3/6	5/6	5/6	4/6	5/6
200	6/6	6/6	4/6	5/6	5/6	5/6	6/6	5/6	6/6

* Prepared on synthetic medium

BV = Ben Venue lots

CSC = Commercial Solvents lots

units per 20-gram mouse is shown. In general, it will be noted there is fairly good correlation between the size of the dose and the incidence of renal damage. Analysis of these data indicates that there is no evidence of statistically significant differences between the material obtained with surface culture bacitracin and deep-tank culture. Lot B-481102 prepared on synthetic medium and grown by the deep-tank-culture method appears to be the most toxic of all the deep-culture material.

In Table III the severity of the renal injury is shown for the various lots of bacitracin and the various dosage levels employed. It is apparent that there is no statistically significant difference in the various lots with the exception of Lot B-481102 prepared on synthetic medium. This is of interest in relation to the use of these various lots clinically. Lot B-480420 has been widely employed by a number of highly competent clinical investigators who have all indicated that it has shown a very low clinical toxicity. On the other hand, B-480210 and B-480512 were reported to be

too toxic for parenteral administration. The laboratory studies in mice indicate that there is very little, if any, difference in the amount of renal damage produced in these animals by the several lots of bacitracin.

Through the cooperation of Dr. Frank L. Meleney of Columbia University, Miss Barranovsky, statistician and secretary for the bacitracin study, has supplied clinical data on the patients treated with these specific lots of bacitracin. An attempt to correlate these data with the mice data is shown in Table IV.

In Dr. Meleney's interim report (2), he stated that the dosage used with CSC deep-culture material in general was higher than that employed with the original surface culture product. In comparing the clinical toxicity of these two types of material, he states, "It is true that the dosage with the CSC lots was somewhat larger except for Lot 480420, but this cannot account for all of the difference." These animal studies indicate that the dosage may well be responsible for any differences noted in degree of clinical toxicity.

TABLE IV

The severity of nephrotoxicity in man when treated intramuscularly with bacitracin

- 0 = No more than +1 albumin, not over 5WBC/H.P. field, no R.B.C., occ. gran. cast.
 +2 = Transient albuminuria +2 to +3, WBC = 5 to 25/H.P. field, rare R.B.C. moderate numbers of gran. casts, no prolonged rise in T.N.P.N. or BUN, no prolonged fall in P.S.P.
 +4 = Persisting nausea and vomiting, prolonged rise in T.N.P.N. and BUN, prolonged fall in P.S.P., +4 albumin, many casts, R.B.C., W.B.C., or cases in which treatment had to be stopped because of oliguria or rash.

Total dose units	BV* 140	BV 142	BV 144	CSC B480420	CSC B480210	CSC B471212
40,000-80,000	0	—	—	+2, +2	—	—
80,000-200,000	—	0,0,0,+2	—	0,+2	—	—
200,000-300,000	—	+2,0,0	—	0,+2,+2,+2	—	+2,+4
300,000-400,000	—	—	—	+2,0	+4	—
400,000-500,000	—	—	—	—	+4	0,+2
500,000-600,000	—	0	—	—	—	+2
600,000-700,000	—	+2	—	0	—	—
700,000-800,000	—	—	—	—	—	+4
800,000-900,000	—	—	—	—	—	—
900,000-1,000,000	—	—	—	—	+2	—
1,000,000-1,300,000	—	—	—	—	—	0,+2
<i>Mouse data</i>						
Nephrotoxic value	1.10	0.91	0.95	1.25	1.32	1.11
No. of mice with renal injury	15	11	10	12	14	13
No. mice treated	18	18	18	18	18	17

* See abbreviations Tables I and II

DISCUSSION

Renal pathology

The renal pathology associated with the parenteral administration of bacitracin is, of course, one of the disturbing problems associated with the widespread use of this highly effective antibiotic. It is well known that bacitracin is a complex substance, presumably polypeptide in nature, containing a large number of amino acids, several of them in significant quantities in the *d* form. Evidence to date points toward the fact that bacitracin as presently constituted is a relatively pure substance and that this nephrotoxicity may well be inherent in the molecule. There are, however, some encouraging indications that this toxicity may at least be reduced materially if not entirely eliminated.

The pathology is distinctly one of lower nephron involvement. The changes are those of degeneration of the lining tubular epithelium, its subsequent desquamation and the formation of hyaline casts. This may proceed to a point where the major portion of the tubules become blocked. In most animals surviving a 72-hour period, evidence of regeneration of the tubular epithelium can be demonstrated. In a few clinical cases of terminal

infection where bacitracin therapy was employed, similar changes were observed. Some of these cases show evidence of associated renal pathology so that it is far from certain that the bacitracin has been a factor in the death of these patients. Most of these fatal cases were suffering from subacute bacterial endocarditis caused by organisms not sensitive to either penicillin or streptomycin. Preliminary observations suggest that a synergistic relationship exists between penicillin and bacitracin in infections of this type. This may open up a field of usefulness for bacitracin in dosage levels sufficiently low to permit its use in combination with other antibiotics without fear of the development of serious renal damage.

It is apparent from these comparative studies that very little correlation exists between the gross and microscopic pathologic changes in mice. It is likewise true that no correlation exists in the degree of renal change in the several mice to whom the same dose of bacitracin was administered parenterally. Similar differences in toxicity have been shown to exist in different strains of mice. Thus it would be theoretically possible to supply data relating to the LD-50 dosage which would not only be acceptable by present FDA specifications but would also meet with the tentative requirements

suggested by the clinicians if mice from a resistant strain were employed. The obvious conclusion must be reached that the mouse is an unsatisfactory test animal for nephrotoxicity.

SUMMARY AND CONCLUSIONS

Comparative studies of the nephrotoxicity of bacitracin when grown by surface and deep-tank culture are presented. No significant statistical differences in nephrotoxicity were found with the various lots of bacitracin.

A discussion of the renal pathology reveals that the lesion is one affecting the lower nephron.

In animals surviving a 72-hour period, representing the time interval required for evidence

of acute toxicity, tubular regeneration is apparent indicating that the changes are reversible.

There is so little correlation between the gross and microscopic evidence of renal pathology that it must be concluded the mouse is an unsatisfactory test animal.

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CHEMICAL STUDIES ON POLYMYXIN B¹

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AND ALBERT E. TIMRECK

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Polymyxin is a generic term for chemotherapeutic antibiotics elaborated in fermentations of various media by strains of *Bacillus polymyxa* (1). The known polymyxin polypeptides (A, B, C, D, and E) have closely similar chemical and biological properties, and have in common L- α , γ -diaminobutyric acid, L-threonine and an unidentified C₉ optically-active fatty acid. In addition to these acids, polymyxin A (aerosporin) contains D-leucine (2). Polymyxin A, first described by Ainsworth, Brown and Brownlee, was isolated by Brownlee and Bushby, and Catch and Friedmann (3) from broth cultures by adsorption onto a suitable carbon and elution with aqueous acetone containing sulfuric acid. Further purification was obtained by converting the antibiotic to the helianthate and subsequently to the hydrochloride. Polymyxin D, which is composed of the constituents of polymyxin A plus serine, was described by Benedict and Langlykke (4) and Stansly *et al.* (5). It was purified by adsorption onto carbon after clarification of the fermentation liquors, elution with acid-methanol and precipitation with acetone. Further purification by Shepherd *et al.* (6) involved butanol extraction, fractional precipitation of the picrate and conversion to the hydrochloride. Hydrolyzates of polymyxin B (7), another basic polypeptide produced by strain CN 1419 of *B. polymyxa* (Wellcome Foundation Culture Numbers), show the presence of D-leucine and phenylalanine as well as the three constituents common to all the polymyxins.

During the course of studies on polymyxin B designed to eliminate toxic impurities and prepare high potency material, a procedure was developed involving adsorption of the active principle on cotton sodium succinate, elution with dilute sulfuric acid, precipitation of the antibiotic with 1-(4-chloro-o-sulfiophenyl)-5-hydroxy-3-

methyl-4-(p-(p-tolylsulfonoxo)-phenylazo)-pyrazole (Polar Yellow), liberation of the antibiotic, precipitation as its free base and conversion to the acid salt. The antibiotic was subsequently crystallized as its naphthalene β -sulfonate by a procedure described by Wilkinson (8).

The homogeneity of material from each purification stage was studied by partition chromatography on paper and by chemical and biological means. The paper partition chromatographic studies were carried out by a method similar to that described by Goodall and Levi (9) for penicillins. After developing and drying the paper strips, they were placed on the surface of nutrient agar previously seeded with *Escherichia coli* instead of *Bacillus subtilis*. In this way, the positions of the zones of inhibition established the presence of another active substance in certain polymyxin B fractions.

Crude material obtained from the cotton sodium succinate column was found to produce a histamine-like lowering of the blood pressure when injected into anesthetized cats. The histamine-like substance was removed from the polymyxin B by purification through its Polar Yellow salt. Fractions of moderately high potency (*ca.* 6500 units/mg.), obtained by a dual purification through the Polar Yellow salt, showed two spots on the chromatogram indicating the presence of an active minor component associated with the polymyxin B. When the acid hydrolyzates of each of these active materials were chromatographed, however, identical amino acid spectra were obtained.

In addition to the recognized components of polymyxin B, an unknown substance was routinely found high in the solvent band of the paper chromatogram of hydrolyzates of this material. Consideration of the R_F differences between glycine and alanine, alanine and valine, valine and leucine, and leucine and the unknown patch, 0.2, 0.15, 0.15, 0.1, respectively, led to the suggestion that the substance might be a C₇-amino acid. Consequently, the three leucine homologs RCH₂-

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CHNH_2COOH were synthesized in which the R groups were $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$, $-\text{CH}_2(\text{CH}_3)-\text{CHCH}_3$ and $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$. Chromatography of these known C₇-amino acids showed them to resemble the unknown patch, but none was identical with it. In a like manner, a similar sample of polymyxin hydrochloride separated into nine fractions by alumina chromatography showed that the hydrolyzates of all of these fractions were indistinguishable from the products of pure polymyxin B. The minor component produced by the strain was eliminated by adding alkali and precipitating the free polymyxin B base. After separating the insoluble antibiotic, the presence of the extraneous substance could be demonstrated by chromatographing the filtrate on paper. Materials of high biological activity purified through the free base followed by crystallization of the naphthalene β -sulfonate salt (8) showed but a single active component by partition chromatography.

METHODS

Paper chromatography. An apparatus for the partition chromatography method using paper was designed after suggestions of Consden *et al.* (10) and Goodall and Levi (9). The method of paper chromatography using ascending flow, described by Williams and Kirby (11), was adapted to the work discussed here by using the butanol-15% acetic acid-water solvent system and developing in one dimension only. Both intact materials and hydrolyzates were similarly analyzed on paper (12).

Hydrolysis of all samples was carried out by a standard procedure. The sample was dissolved in 5 N HCl (5 to 10% W/V) and heated for 2.5 hours in a sealed tube placed in a steam bath. This solution was evaporated to dryness in an open dish on a steam bath, and the residue was dissolved in water so that the stock solution prepared for chromatography corresponded to a concentration of 100 $\mu\text{g.}/\mu\text{l.}$ of intact material.

Solutions to be chromatographed were placed on spots along a line 2 inches from the edge of a sheet of Whatman No. 1 paper in amounts containing 10 to 100 $\mu\text{g.}$ of intact or 500 to 1000 $\mu\text{g.}$ of hydrolyzed polymyxin B. After drying, the sheet was suspended in the chamber, inoculated edge down, for at least one hour before starting the development, so that the paper could come to equilibrium with the atmosphere of the chamber. The lower edge of the paper was then placed in a trough containing butanol-acetic acid-water mixture and the chromatogram was developed by an ascending solvent flow for about 15 hours. The developed sheet was dried in an oven at 60° and then sprayed with a 0.25% solution of ninhydrin in acetone. Re-tying for five minutes to react in the amino acid-ninhydrin color reaction and the R_F values of the amino acids obtained from hydrolyzates of poly-

myxin B fractions was made with known acids and with known acids added to the test material.

Biological assay. In these studies, the method of biological assay was essentially that developed by Stansly and Schlosser (13) and modified by Benedict and Stodola (14) using *Brucella bronchiseptica*, strain NRRL B-140. A polymyxin hydrochloride, containing 6,650 units/mg., supplied by the Wellcome Research Laboratories (England), was used as a standard.

The paper chromatograms of intact materials were further examined for antibiotic activities by drying at 50°, after development, and pressing on agar plates (13" \times 17") seeded with *E. coli*. After refrigerating at 0° for two hours, the paper was removed from the surface of the agar and the agar plate was incubated at 37° for 15 hours. Active substances in certain samples were located by the zones of inhibition of the organism.

EXPERIMENTAL

Clarification of fermentation broth. Eight liters of a freshly harvested broth was adjusted to pH 2 with dilute sulfuric acid and heated at 90° C. for 30 minutes so as to coalesce the cells and thin out the mucilage. About 60 gm. of Super-Cel was added and the mixture was filtered. The clarified filtrate showed no appreciable loss of activity.

Adsorption of polymyxin on cotton sodium succinate. Cotton acid succinate (15) was prepared by dissolving 25 gm. of fused sodium acetate and 200 gm. of succinic anhydride in 1,500 ml. of glacial acetic acid and submerging 50 gm. of cotton in this solution at 100° for 48 hours. The partially esterified cotton was filtered, washed with water, with dilute hydrochloric acid and finally thoroughly with water. The cotton acid succinate was found to be a poor cation exchange medium for polymyxin. Therefore, the cotton acid succinate was converted to cotton sodium succinate by suspending the former in water and titrating with dilute sodium hydroxide to a phenolphthalein endpoint. After filtering and washing with water, the product was dried *in vacuo* at 50° for 24 hours.

Eight liters of the clarified polymyxin B broth (2,000 units/ml.) was poured into a column containing 50 gm. of cotton sodium succinate. About 4% of the original activity in the polymyxin B broth was found in the column. The column was washed with 2,000 ml. of water. The column was then washed with 2,000 ml. of water and the product was eluted from the column with 50 ml. of 0.25 M sodium chloride. The product was precipitated by the addition of

the polymyxin B eluted from the cotton sodium succinate column contained a histamine-like substance which was detected by the lowering of the blood pressure in cats.

Isolation of polymyxin B using Polar Yellow. The histamine-like substance could be removed from the polymyxin B eluate by purification through its Polar Yellow salt. Hence, the acid eluate was neutralized to pH 3.5 with sodium hydroxide and treated with 18 gm. of Polar Yellow. After stirring the precipitate for 15 minutes, the mixture was filtered, washed well with water and dried *in vacuo* at 50° for 48 hours. The dried mixture was suspended in 1 liter of a mixture of 85% acetone-15% methanol and treated with 25 ml. of a solution of triethylamine sulfate (0.45 gm./ml.) in methanol in order to convert the polymyxin B into the methanol insoluble sulfate. Af-

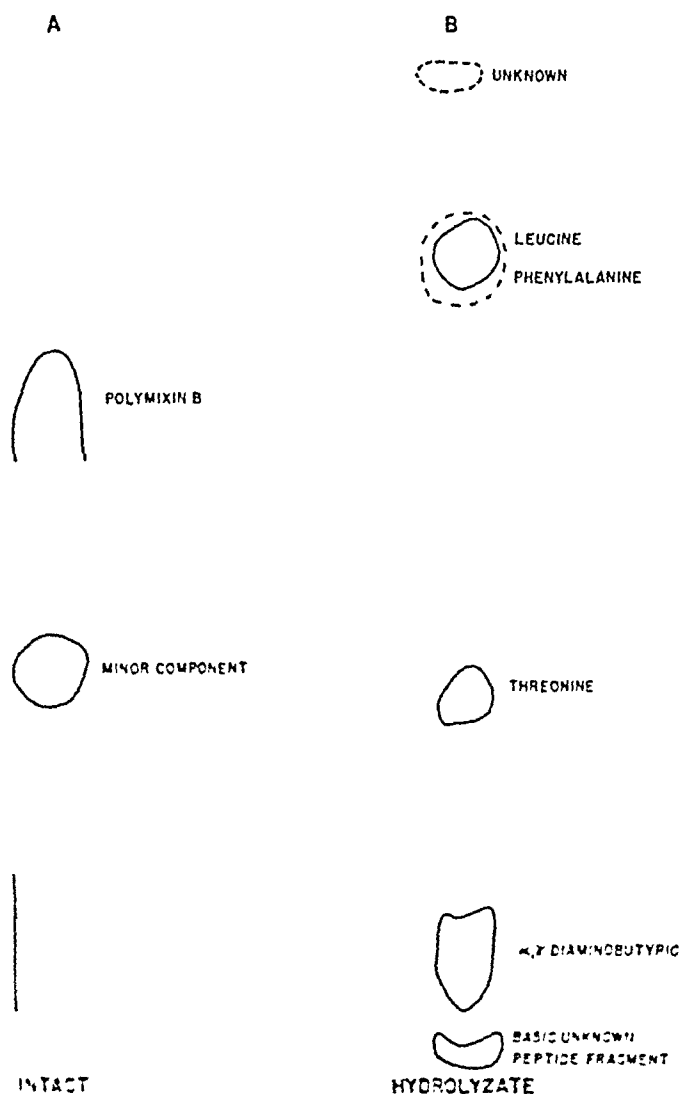


FIG. 1

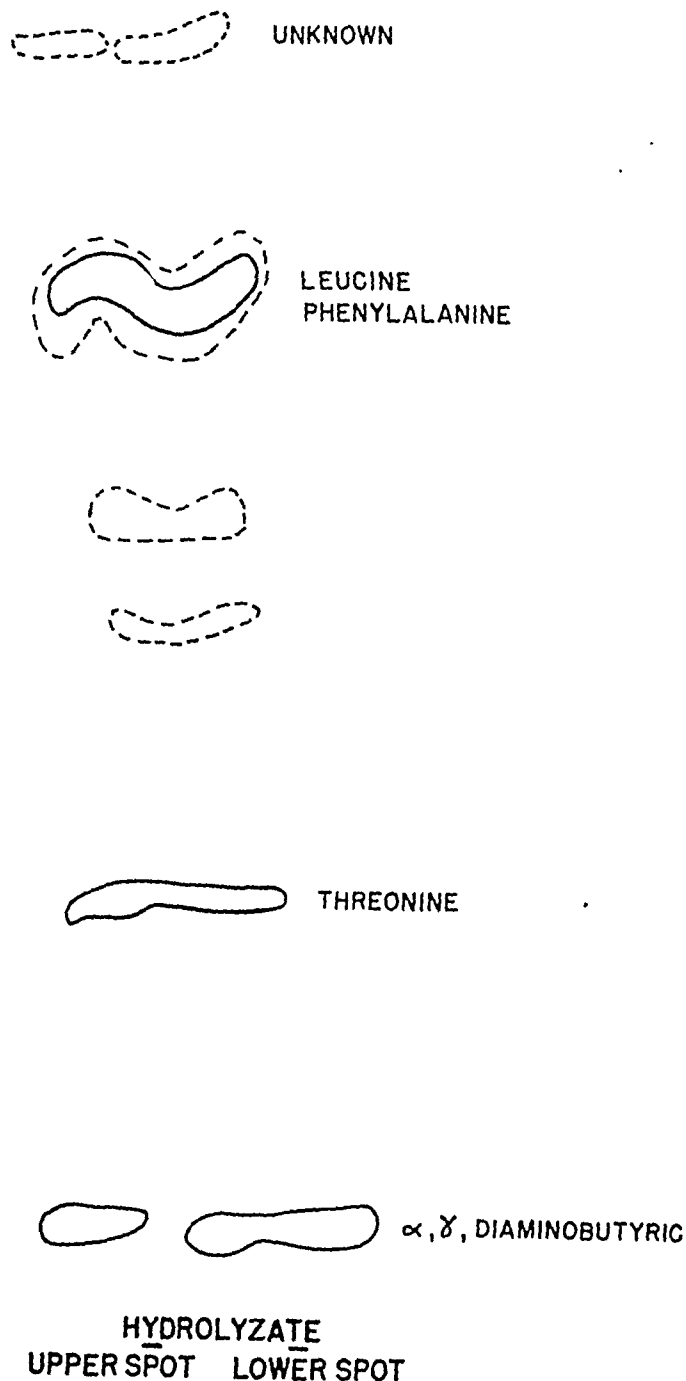


FIG. 2

ter it was shaken for one hour, the mixture was filtered, the precipitate was washed with acetone-methanol mixture and finally with acetone. After it was dried, the cake was triturated in 500 ml. of water, filtered, and washed with water. The colorless filtrate was vacuum freeze-dried; a yield of about 75% of the active substance was obtained. The product had a potency of 5,200 units/mg. when assayed against *B. bronchiseptica* and had 2.3% ash.

Partition chromatograms of this intact material

showed a spot corresponding to the R_F value of polymyxin B and in addition the presence of a biologically active lower second patch (Figure 1A). However, the hydrolyzate of the intact material showed only the presence of the expected components (Figure 1B).

In the course of investigating the contaminant, a comparatively large amount of the material was put onto chromatographing paper as a swath instead of individual spots. After the usual 15-hour development, a very small portion of this swath was ninhydrin-treated to locate the spots. The remainder of the paper was cut horizontally so that two strips were obtained, each holding one component. The material held on each paper was eluted and hydrolyzed separately. It was found, on chromatographing the hydrolyzates, that both spots gave identical amino acid spectra (Figure 2).

Attempts to isolate enough of the active extraneous material for use in determining its contribution to the toxicity in animals were made by studying the chromatography of the substances on alumina.

Brockmann alumina was treated with 50% sulfuric acid to pH 6.0, backwashed with distilled water until the washings were sulfate-free and dried 24 hours at 100° C. The dried alumina was transferred to the column as a slurry in methanol. After the column was packed, a solution of polymyxin B hydrochloride (60,000 units/ml.) in anhydrous methanol was carefully poured into the column. The chromatogram was developed with absolute methanol, 95%-, 90%-, 80%-, 50%-methanol, water, and finally with 0.1 N and 0.5 N sulfuric acid. After the third fraction, each solution leaving the column was collected until it showed less than 500 units/ml., whereupon the chromatogram was developed with the succeeding more dilute methanol. Twelve fractions were collected; the methanol was evaporated from each and the residue was vacuum freeze-dried. The results of the column are summarized in Table I.

Portions of the first nine alcohol-developed fractions were redissolved and chromatographed intact on paper. The purest fraction (No. 1) appeared to be composed only of polymyxin B while the lowest potency fraction (No. 4) seemed to be made up mainly of the minor component produced by this strain. After going through a minimum, fractions 6, 7, and 8 appeared to be composed

TABLE I
Fractions of polymyxin B from alumina column

Fraction number	Developing solution	Potency units/mg.
1	100% methanol	6750
2	100% methanol	5350
3	100% methanol	4150
4	95% methanol	3530
5	90% methanol	4750
6	80% methanol	5750
7	80% methanol	5750
8	50% methanol	6050
9	50% methanol	3700
10	water	2040
11	0.1 N sulfuric acid	—
12	0.5 N sulfuric acid	40

mainly of polymyxin B. Further examinations showed that the hydrolyzates of all of these fractions yielded identical amino acid spectra, indicating that the two active substances are qualitatively related (Figure 1A). This evidence is based upon the relative solubilities of the active components in two solvents, and in turn, on their relative speeds on two chromatograms. There was found to be some important difference between the active substances because the lower component appears to be only weakly active biologically. The most effective technique for eliminating the second component involves the precipitation of polymyxin B as its free base. This was achieved by the use of amines, ammonia, sodium hydroxide, etc.

The purification of polymyxin B by precipitation of its free base. Thirteen grams of polymyxin B, obtained from the Polar Yellow purification, were dissolved in 200 ml. of water. To the cooled solution (0° C.) was added an aqueous solution of triethylamine until no further precipitation occurred (pH 11.0). The polymyxin-B-free base was filtered over Super-Cel, washed with ice water and finally with ether until it was free of triethylamine. The base was suspended in water and dissolved by the addition of dilute sulfuric acid (pH 2.5), and warming to 50°. It was neutralized (pH 5.5) with barium hydroxide and filtered from the barium sulfate. The clear filtrate was vacuum freeze-dried: yield 85%; potency 7200 units/mg.; ash 0.5%. The optical rotation increased from -61° to -76° ; and on chromatographing it on paper only one spot was observed corresponding to the R_F value for polymyxin B. The filtrate from the triethylamine precipitation was also vacuum freeze-dried after neutralization to pH 5.5; only about 10% of the activity was

found in this portion. Paper chromatography showed this intact material to be composed mainly of the lower active component.

Crystalline polymyxin B naphthalene β -sulfonate. This crystalline salt was prepared (8) by suspending 12 gm. of polymyxin-B-free base in 200 ml. of water, and adding a solution of naphthalene β -sulfonic acid with constant stirring to pH 5. The sulfonate was precipitated as a heavy oil. After standing, the supernatant liquid was decanted. The oil was washed three times with 150 ml. portions of water and dissolved in the minimum amount of ethanol. Additional alcohol was added until the solution appeared turbid whereupon it was allowed to stand at 5° for 18 hours. The sulfonate precipitate was filtered and washed with small portions of ethanol. It was then suspended in about 250 ml. of ethanol at 50° C. and treated with water until the sulfonate completely dissolved. The solution was stirred with 1 gm. of Darco G-60 at 50° for one-half hour and filtered. Ethanol was then added to the decolorized solution until cloudiness appeared, and the solution was allowed to cool slowly. The white, crystalline sulfonate crystallizes as very fine needles. The sulfonate was recrystallized by redissolving the crystals and repeating the procedure described above. Analysis of polymyxin B naphthalene β -sulfonate (dried *in vacuo* at 100° C. at 0.1 mm. Hg): C, 49.11%; H, 6.81%; S, 6.31%; N, 13.41%; $[\alpha]^{25}_D = -63.3^\circ$ (c, 1% in 75% ethanol in water); m.p. 235–8° C. with decomposition.

The crystalline polymyxin B naphthalene β -sulfonate was converted to the hydrochloride by suspending the sulfonate in anhydrous methanol and adding concentrated hydrochloric acid until complete solution took place. Polymyxin hydrochloride was precipitated by pouring the acidified methanol solution into six volumes of acetone since polymyxin B naphthalene β -sulfonate is completely soluble in this mixture. The polymyxin B hydrochloride, after drying, showed $[\alpha]^{25}_D = -75.7^\circ$ (c, 1% in 75% ethanol in water).

Partition chromatography on paper of the naphthalene β -sulfonate and the sulfate produced by metathesis reaction consistently showed a single component. Acid hydrolyzates of this component showed the presence of threonine, leucine, phenylalanine, α,γ -diaminobutyric acid, and a C_6 optically active fatty acid.

Acid hydrolysis of crude and crystalline materials yielded an optically active acid previously isolated and described by Catch, Jones and Wilkinson (2). After heating in concentrated hydrochloric acid, the fatty acid component was isolated by extraction with ether and distillation of the solvent. The yield of fatty acid was approximately 5% by weight of intact polymyxin of 5,000 units/mg. The crude acid was distilled *in vacuo* under nitrogen, and a clear colorless product was obtained with the following properties: b.p. 117–120° C. at 7 mm.; n 1.4307; $[\alpha]^{25}_D = +32^\circ$ (c, 1% in methanol); sp. gr. 0.93; mol. wt. (sodium hydroxide titration) 157.3. Anal. Calcd. for $C_9H_{18}O_2$: C, 68.4; H, 11.4. Found: C, 68.2; H, 11.4. Amide: m.p. 96° C. p-Bromphenacyl ester: m.p. 60° C.; $[\alpha]^{25}_D = -25^\circ$ (c, 1% in methanol). This optically active isomer of pelargonic acid has been recently shown to be *d*-6-methyloctan-1-oic acid by Wilkinson (8).

SUMMARY

A method for the recovery, purification and crystallization of polymyxin B is described in which the fermentation broth is clarified, adsorbed on cotton sodium succinate, eluted, precipitated as the polymyxin B Polar Yellow salt, converted to the free base and crystallized as the naphthalene β -sulfonate salt. Purification through the Polar Yellow salt removes a histamine-like contaminant, but does not remove a second minor active component the presence of which was demonstrated by partition chromatography on paper. The extraneous active substance is soluble in excess of base and can be separated from the insoluble basic polymyxin B. Paper chromatograms of the crystalline polymyxin B naphthalene β -sulfonate show a single component. The composition of the hydrolyzates of the polymyxin B and the minor active constituents are qualitatively related since they show the same acid spectra: Threonine, leucine, phenylalanine, α,γ -diaminobutyric acid, and a C_6 optically active fatty acid.

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LABORATORY AND CLINICAL STUDIES OF POLYMYXIN B AND E^{1,2}

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The antibiotics, polymyxin A B C D and E, have been described in the recent literature. Their antibacterial spectra are similar. They differ from one another in amino acid content. It is the purpose of this presentation to describe our studies of the antibacterial activity, pharmacology, untoward reactions, and clinical experience with polymyxin B and polymyxin E.⁵

IN VITRO ACTION

We found that the susceptibility to polymyxin of 78 strains of ten different genera by the tube dilution test (Table I) is marked against *Salmonella*, *Shigella*, *Klebsiella*, microorganisms of the *coli aerogenes* groups, and most importantly, *Pseudomonas*. *Brucella* and many staphylococci are moderately sensitive, while *Proteus* and hemolytic streptococci are refractory. It is of interest that a strain of *Pseudomonas*, which developed high resistance to streptomycin in three daily transfers, did not become resistant to polymyxin after 27 transfers. Size of inoculum and human serum reduced but slightly polymyxin activity. The concentration of polymyxin required to inhibit *Klebsiella* and *Pseudomonas* is not reduced significantly by the addition of subinhibitory amounts of streptomycin, aureomycin, sulfadiazine or penicillin, either singly or in several combinations. Polymyxin antagonizes the cumulative streptomycin-penicillin action on *Proteus*.

The administration of polymyxin to patients in divided doses by the intramuscular route in a daily dosage of 2 to 4 mgm. per kilogram of body weight results in blood serum concentrations of 1 to 8 μ g. per milliliter. The peak occurs 30 minutes to two hours following injection; one-half the peak level is noted at six hours, and the drug can be detected in blood serum up to 12 hours. When the injections are repeated at six-hour intervals, blood serum levels in excess of 30 μ g. per milliliter may be obtained after four doses. No cumulative increase in blood levels has been noted when the

TABLE I
Sensitivity to "aerosporin" polymyxin B

Organism	No. strains tested	Sensitivity range in μ g./ml.	Mean sensitivity μ g./ml.
<i>Salmonella</i>	5	0.08- 0.3	0.18
<i>Shigella</i>	7	0.08- 0.6	0.3
<i>Klebsiella</i>	5	0.16- 1.2	0.48
<i>Coli-aerogenes</i>	17	0.06- 1.6	0.8
<i>Pseudomonas</i>	19	0.3 - 3.1	1.2
<i>Paracolonobacter</i>	2	1.2 - 1.6	1.4
<i>Staphylococcus</i>	6	1.2 - 5.0	2.0
<i>Brucella</i>	8	0.6 -12.0	4.1
Beta hemolytic streptococcus	6	3(1).->12.0	>12.0
<i>Proteus</i>	5	100.->100.	>100.
Total	78		

drug is administered every 12 hours in a dosage of 1 mgm. per kilogram. Polymyxin is excreted in the urine less rapidly than penicillin and streptomycin. The amount detected in the first 12 hours after injection is less than 0.1% of the injected dose. After 12 hours, excretion increases progressively (Figure 1). On a dosage of 3 mgm. per kilogram of body weight per day levels in the urine range between 40 and 400 μ g. per milliliter after 24 hours of therapy.

We have noted that less than 1% polymyxin passes through Zeitz or sintered glass ultra fine filters. It is practical to sterilize urine for assay by heating the sample to 60° C. for one hour.

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⁴ Captain, M.C., United States Army.

⁵ Kindly supplied by Burroughs Wellcome and Company.

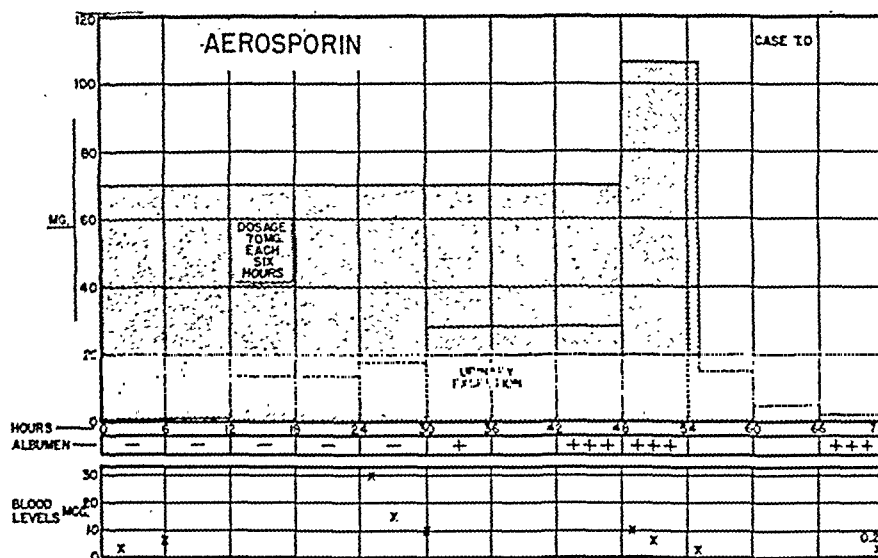


FIG. 1

listed as benefitted, there was no bacteriological relapse up to three weeks after cessation of treatment. The most dramatic responses are obtained in polymyxin-sensitive, acute pseudomonas pyelonephritis. Usually, 12 hours after polymyxin therapy is begun the infection appears under control. The results of therapy in seven of the 20 patients are considered doubtful, either because there was little symptomatic improvement, or the urine was not rendered completely or permanently bacteria-free. In three of the 20 patients treatment was regarded as an outright *failure*. Naturally drug-resistant organisms were present in one and multiple cortical abscesses in scarred kidneys in the other two.

CLINICAL EXPERIENCES

The therapeutic effectiveness of polymyxin has been tested at Brooke General Hospital in a series of patients with infections of the urinary tract and infections in granulating wounds. Also, the drug orally administered has been evaluated as an intestinal antiseptic in preparation of the bowel for surgery.

Twenty patients with severe urinary tract infections were treated with polymyxin intramuscularly administered over periods of one to six days in dosages of 2 to 5.6 mgm. per kilogram of body weight per day, divided in two, four, or six doses. The average, and we now believe the optimum dose, was 2.5 mgm. per kilogram per day divided in four doses for three days. Pain at the site of injection was common, but obviated by solution of the drug in 1% aqueous procaine. Nineteen of the 20 patients had extended previous trials of other chemotherapy. Ten of the 20 patients were regarded unequivocally as benefited. Improvement in most instances was observed after 24 hours, as manifested by marked reduction or elimination of bacteria from the urine, regression of symptoms, decrease in fever and gross and microscopic improvement in the urine. In all patients

It is of importance that drug resistance did not develop in any of the cultures of these patients. There can be little doubt of the therapeutic effectiveness of polymyxin for the treatment of urinary tract infections produced by susceptible gram-negative bacilli, particularly acute pseudomonas pyelonephritis.

Polymyxin orally administered does not produce detectable blood levels. It exerts a rapid bacteriostatic action on most coliform bacteria, as well as on some types of cocci. Solutions of the drug were administered by mouth to 14 subjects in doses of either 200 or 400 mmm. per kilogram per day. All coliform organisms—except *Proteus*—were suppressed in these 14 subjects within 24 to 72 hours (Figure 2). Cocci were instantaneously reduced in

number, while *Clostridia* and *Monilia* were unaffected. Suppression of coliforms was maintained for 11 to 24 days, at which times the drug was withdrawn. Coliform organisms returned one to six days following cessation of therapy. In only one subject ingesting 2.5 mgm. per kilogram per day did the *E. coli* reappear in the stools during treatment. This organism was sensitive to 0.78 μ g. per milliliter of polymyxin. Continuation of therapy for five additional days eliminated these bacteria. All coliform organisms tested were sensitive to polymyxin before and after therapy. It is apparent at this stage of the study that 200 mgm. a day is a minimally effective dose, and that 400 mgm. is a more consistently suppressive dose.

Several granulating wounds infected with *Ps. aeruginosa* were treated by the topical application of 1% polymyxin in salt solution or carbowax base, with eradication of the organisms in all instances, except where devitalized tissue was present.

TOXICITY

No patients in this series developed any of the usual drug sensitivity reactions. No evidence of toxicity was noted following topical or oral administration of the drug. In the case of intramuscular injection, in nearly every instance neurologic disturbances were present, as manifested by paresthesias and hypesthesias mainly about the face and scalp, mild dizziness and weakness.

These symptoms usually appeared with the first dose, and persisted throughout the course of treatment. The only objective neurologic abnormality was circumoral flush. Neurotoxic symptoms disappeared in every instance within 24 hours after the last dose was administered. In no case were the symptoms severe enough to warrant cessation of therapy.

When the daily intramuscular dosage did not exceed 2.5 mgm. per kilogram, there was no increase in the blood urea nitrogen or non-protein nitrogen, nor was there any clear-cut evidence of nephrotoxicity in the form of tubular damage attributable to the drug. It is reiterated that most subjects given the drug initially had abnormal urines. Patients with normal urines did not present nephrotoxicity after three to five days on a dosage of 2 mgm. per kilogram of body weight. When the dosage was 4 mgm. per kilogram or more, tubular damage was usually noted, as evidenced by increased albuminuria, red cells, white cells, renal cells and inconstantly, granular casts in the urine. Oliguria and fixation of the specific gravity at a low level were not demonstrated in any of our cases. Urinary abnormalities produced as a result of drug therapy disappeared within four days after the drug was stopped. Both polymyxin B and polymyxin E are essentially similar in their toxic effects. We are encouraged by the relative absence of neurotoxicity in a recently tested batch of polymyxin E (EA 113).

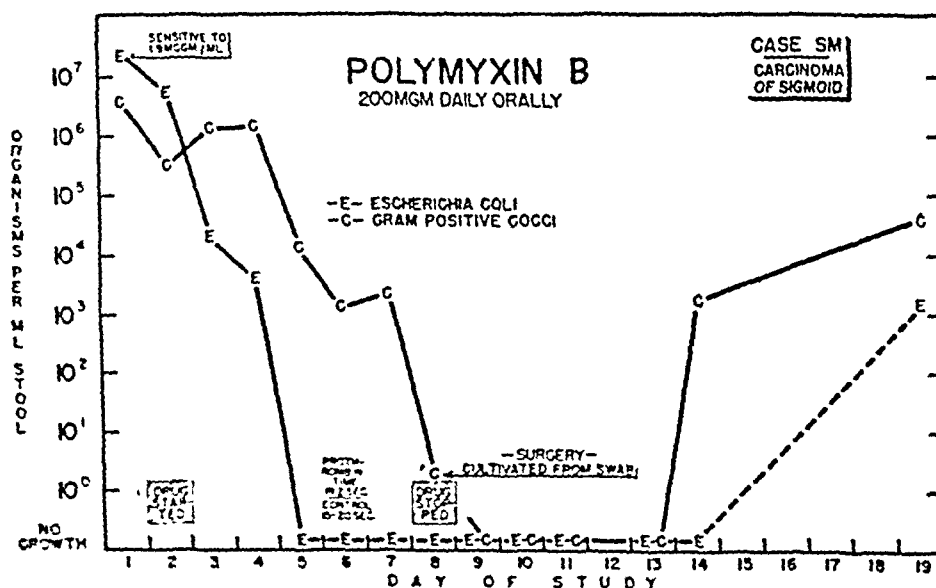


FIG. 2

SUMMARY

Polymyxin B and polymyxin E are potent chemotherapeutic agents against susceptible gram-negative urinary tract infections, including those produced by *Ps. aeruginosa*. They are useful topically applied in eradicating these organisms in granulating wounds. Polymyxin is a potent intestinal antiseptic. Drug resistance of bacteria incident to therapy does not occur. Certain reversible neurotoxic phenomena occur following intramuscular injection of the drug, both with polymyxin B and polymyxin E, even with a single 50 mgm. dose. When the daily dosage exceeds 2.5 mgm. per kilogram, intramuscularly administered every six hours, nephrotoxicity is encountered.

Further toxicity studies on patients receiving 2 mgm. per kilogram per day intramuscularly are in progress.

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PREPARATION OF A MODIFIED SUBTILIN SUITABLE FOR THE TREATMENT OF TUBERCULOSIS AND OTHER INFECTIONS IN ANIMALS^{1, 2}

By A. J. SALLE AND GREGORY J. JANN

(From the Department of Bacteriology, University of California, Los Angeles)

Subtilin is an antibiotic extracted from the cells of a particular strain of *Bacillus subtilis*.^{3, 4} It is a low molecular weight substance that dialyzes through collodion membranes, is sensitive to light and alkali, and has the structure of a peptide.

Subtilin is soluble to at least 30 per cent in salt-free acidified solution. The solubility in acid solution is markedly decreased by the presence of salt. In the presence of 0.5 to 1 per cent of sodium chloride the solubility is reduced to approximately 0.5 to 0.2 per cent. The addition of serum to an aqueous solution of subtilin produces a marked precipitation. The antibiotic is probably a basic substance giving water-soluble salts (1).

The antibacterial spectrum of subtilin (2) is similar to that of penicillin. It seems reasonable to postulate that the antibiotic might prove useful in infections caused by penicillin-fast or penicillin-resistant organisms. In addition, subtilin is effective against *Mycobacterium tuberculosis* which is not antagonized by penicillin.

The low solubility of subtilin in salt solutions and in serum is probably a desirable property in the treatment of infections caused by such organisms as the pneumococcus (3), streptococci (4), micrococci (5), the anthrax bacillus (6), etc., in which relatively small doses are required. Precipitation of the antibiotic in the presence of body fluids should slow down its elimination, thus giving

ing a more prolonged effect. However, for the effective treatment of tuberculosis the antibiotic concentration of the tissues must be maintained at a very high level. Unless the solubility of subtilin in the presence of serum can be increased it will be of limited value in the chemotherapy of tuberculosis.

EXPERIMENTAL

The solubility of the antibiotic may be improved considerably as follows: 1. Dissolve 1.5 gm. of subtilin in 50 ml. of 20 per cent urea in distilled water; 2. dissolve 0.1 gm. of pectin in 50 ml. of distilled water; 3. mix 1. and 2. and let stand overnight at room temperature for a precipitate to settle out. Centrifugate the mixture and decant the clear supernatant liquid. This clear liquid contains subtilin that is soluble in serum in all proportions and may be administered without any further treatment.

The injection of unmodified subtilin subcutaneously resulted in the formation of very hard nodule-like deposits under the skin. The subtilin was apparently largely precipitated. Sometimes these deposits produced an ulceration of the skin followed by discharge of the insoluble subtilin. Obviously, not many injections could be given. The addition of urea to a solution of subtilin increases its solubility and prevents precipitation of most of the antibiotic in the presence of serum. The pectin precipitates out that portion of the subtilin that is not soluble in the presence of serum. The removal of the precipitate gives a serum-soluble subtilin that does not produce nodule-like deposits when injected subcutaneously but is completely absorbed.

The project on the chemotherapy of tuberculosis has been under way for a period of approximately three years. At least 15 experiments have been completed involving a total of over 700 guinea pigs. The work has involved proper dosage, number of doses daily, route of injection of subtilin, and preparation of subtilin before injection.

¹ Presented at the Second National Symposium on Recent Advances in Antibiotics Research held in Washington, D. C., April 11-12, 1949, under the auspices of the Antibiotics Study Section, National Institutes of Health, Public Health Service, Federal Security Agency.

² This work was aided by a grant from the Antibiotics Study Section, National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

³ The culture was originally obtained from Dr. N. R. Smith of the Bureau of Plant Industry, United States Department of Agriculture, Washington, D. C.

⁴ The subtilin used in these experiments was kindly supplied by the Western Regional Research Laboratory, Albany, California.

In most experiments a total of 48 guinea pigs were used weighing approximately 500 gm. each. The animals were infected subcutaneously with 0.3 mg. each of *Mycobacterium tuberculosis* H37Rv grown in Proskauer and Beck's synthetic medium, then divided into four groups. Group 1 animals received subtilin alone; group 2 received streptomycin alone; group 3 received a mixture of subtilin and streptomycin; and group 4 animals served as controls. Treatments were commenced generally the same day that the animals were infected. The antibiotics were injected subcutaneously three times a day at 9 a.m., 3 p.m., and 9 p.m. for 42 consecutive days (six weeks). The results of only the last three experiments are reported here.

Control Guinea Pigs. Animals in this group were inoculated with *Mycobacterium tuberculosis*

TABLE I

Guinea pigs were infected with M. tuberculosis H37Rv but not treated (controls)

Animal	Liver	Lungs	Spleen	Animal	Liver	Lungs	Spleen
A1	++	0	+++	B9	++	+++	++
A2	0	0	++	B10	++	+++	++
A3	0	0	++	B11	0	++	++
A4	++	0	+++	C1	0	++	0
A5	++	++	+++	C2	0	+++	++
B1	+++	++	+++	C3	0	+++	0
B2	+++	0	+++	C4	0	+++	0
B3	+++	+++	+++	C5	0	+++	++
B4	0	+++	+++	C6	++	+++	++
B5	+++	+++	0	C7	0	+++	0
B6	++	0	++	C8	++	++	++
B7	++	0	++	C9	0	+++	++
B8	++	+++	+++	C10	++	+++	++

0 = No macroscopic lesions
 + = Very slight infection
 ++ = More pronounced infection
 +++ = Heavy infection

H37Rv but not treated. They served as the controls. The results are given in Table I. The method of designation of the degree of infection in this and in subsequent tables is as follows: 0 = no macroscopic lesions; + = very slight infection; ++ = more pronounced infection; and +++ = heavy infection. With a few exceptions animals in this group showed pronounced to heavy infections in the liver, lungs, or spleen. A large nodule was present at the site of the original infection.

Animals Treated with Streptomycin. Twenty-six guinea pigs were treated with 9000 units of streptomycin per day (three injections) over a period of six weeks. With a few doubtful exceptions no macroscopic lesions were noted. Animals

TABLE II

Treatment of guinea pigs with streptomycin 2400 units/day (three injections) for a period of six weeks

Animal	Liver	Lungs	Spleen	Animal	Liver	Lungs	Spleen
A1	+	0	0	B9	0	0	+
A2	0	++	++	B10	++	++	++
A3	0	0	+	C1	0	++	0
A4	0	+++	++	C2	0	0	++
A5	0	0	0	C3	+	+++	++
B1	0	+++	0	C4	0	++	0
B2	0	0	++	C5	0	0	0
B3	0	+	+	C6	++	++	++
B4	+	+++	++	C7	0	0	0
B5	+	++	++	C8	++	++	++
B6	0	0	0	C9	0	0	++
B7	+	0	++	C10	+	0	++
B8	++	++	++	C11	0	0	0

0 = No macroscopic lesions
 + = Very slight infection
 ++ = More pronounced infection
 +++ = Heavy infection

appeared normal except for a small nodule at the site of infection.

On the other hand, a similar number of infected animals treated with 2400 units of streptomycin per day exhibited an entirely different picture. The results are reported in Table II. Five of the animals were macroscopically negative. In the others the antibiotic produced only a suppressive effect. Lesions were present in the liver, lungs, or spleen.

Animals Treated with Subtilin. A group of guinea pigs were treated with 22 mg. of subtilin per day (three injections). The results are recorded in Table III. One-half of the animals were treated with a lot of subtilin that exhibited a low tuberculostatic effect; the other half were treated with a quantity of subtilin that showed a high tuberculostatic action. Of the 11 animals in the

TABLE III

Treatment of guinea pigs with subtilin 22 mg./day (three injections) for a period of six weeks

Poor quality of subtilin				Good quality of subtilin			
Animal	Liver	Lungs	Spleen	Animal	Liver	Lungs	Spleen
B1	0	0	0	C1	0	0	++
B2	++	++	++	C2	0	0	0
B3	0	+	0	C3	0	0	0
B4	++	0	++	C4	0	0	0
B5	++	++	+++	C5	0	++	0
B6	0	++	++	C6	0	0	0
B7	0	0	0	C7	0	0	0
B8	++	0	++	C8	++	0	0
B9	++	++	++	C9	0	0	0
B10	++	++	++	C10	0	+	++
B11	++	++	++	C11	0	-	0

0 = No macroscopic lesions
 + = Very slight infection
 ++ = More pronounced infection
 +++ = Heavy infection

TABLE IV

Treatment of guinea pigs with streptomycin 2100 units and subtilin 22 mg./day (three injections) for a period of six weeks

Animal	Liver	Lungs	Spleen	Animal	Liver	Lungs	Spleen
A1	0	0	0	B10	+	0	0
A2	0	0	0	B11	0	0	0
A3	0	0	0	C1	0	0	0
A4	0	0	0	C2	0	0	0
B1	0	0	0	C3	0	0	0
B2	0	0	0	C4	0	0	0
B3	0	0	0	C5	0	0	0
B4	0	0	+	C6	0	+	0
B5	0	0	0	C7	0	0	0
B6	0	0	0	C8	0	0	0
B7	0	0	0	C9	0	0	0
B8	+	0	0	C10	0	0	0
B9	0	0	0	C11	0	+	++

0 = No macroscopic lesions

+

++ = More pronounced infection

+++ = Heavy infection

latter group, five were macroscopically negative. The remaining six exhibited very light infections indicating a pronounced suppressive effect.

Animals Treated with a Mixture of Streptomycin and Subtilin. In this series the animals were treated with both antibiotics. In the earlier experiments the antibiotics were injected separately. In the later experiments it was found that the two antibiotics could be mixed and given in one injection. The results are reported in Table IV. It may be seen that the animals responded excellently to such treatment. Of a total of 26 animals, 21 were macroscopically negative; the remaining five animals exhibited extremely light infections.

DISCUSSION

Subtilin is not a uniform compound but contains a fraction that is insoluble in serum. The removal of this insoluble fraction gives a product that is soluble in serum in all proportions.

It is felt that emphasis should be placed on the results reported in Table III. They indicate that subtilin might well be used alone for the treatment of tuberculosis. It is seen that the animals treated with a good lot of subtilin responded favorably to treatment and that no toxic manifestations were evident despite the massive doses administered. The low toxicity of subtilin *in vitro* is in keeping with its results *in vivo* where it was found that the antibiotic was approximately 20 times more toxic to *Micrococcus pyogenes* var. *aureus* than to

living embryonic chick heart tissue fragments (7).

A number of subtilin samples, prepared and purified at the Western Regional Research Laboratory, United States Department of Agriculture, have been tested for their tuberculostatic potencies. In view of the known impurity of the antibiotic, the samples showed great variation in their action on the tubercle bacillus. However, subtilin prepared and purified by a method which would give consistently good results might well be used in the treatment of tuberculosis.

Another point which we should like to stress is that the antibiotic may be combined with streptomycin in the chemotherapy of tuberculosis, thereby decreasing the dosage of the latter. By so doing, toxic manifestations of streptomycin may be considerably reduced or perhaps eliminated completely.

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ABSTRACTS

Penicillin

Factors Affecting Paper Chromatography of Penicillins.

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Although penicillin chromatography has been used to illustrate the factors discussed, much of the material is equally applicable to chromatography of other antibiotics.

The performance of an analytical chromatogram may be expressed in terms of its resolving power which in turn may be defined as being inversely proportional to the percentage difference in distribution coefficient necessary to produce a given separation of two components. The resolving power of a chromatogram for any pair of components is calculable if certain basic data are at hand. In other words, it is possible to predict whether a given set of chromatographic conditions will effectively separate a given pair of components, or at least to predict under what conditions separation is most likely to occur.

Variation in pH constitutes a convenient method of changing the rate of migration of all components by an equal factor. Change of solvent usually produces an analogous effect. By proper choice of pH and solvent, any desired component may be brought to the region of maximum resolution in the chromatogram. Variation in amount of mobile phase allowed to flow causes a change not only in the position of the components on the chromatogram, but also in their degree of separation. By changing both pH and amount of solvent flow, enhanced resolution may be obtained. Unless excessive flow rates or other faulty techniques are used, the number of theoretical plates realized per unit length of column is probably largely determined by the type of paper chosen.

Comparative Penicillin Assays. D. K. KITCHEN, E. W. THOMAS, C. R. REIN, and W. E. CRUTCHFIELD, JR. Bristol Laboratories, Inc., New York, N. Y.

The purpose of this study was to establish, from a practical standpoint, the degree of accuracy and consistency inherent in two assay procedures. Known standards of crystalline Penicillin G in three concentrations (approximately 3.0, 0.3 and 0.03 units/cc.) in three diluents (gelatine, phosphate buffer and human serum) were prepared. These were assayed by the "cup plate method" using *Sarcina lutea* as test organism and the "serial dilution method" using *B. subtilis* as test organism. Fifteen duplicates were performed using each method at each concentration. All assays were performed immediately following the preparation of the standards from the dry stable salt. The results are graphically portrayed in a table giving numerical values obtained on average blood levels and the percentage of standard measured by each method. A chart depicting individual assay distribution points has been prepared. This demonstrates clearly that under the conditions studied it is apparent that the "cup plate" assay procedure is more accurate than the "serial dilution" method.

The Pathogenesis and Pathology of Airborne Mouse Pneumonitis Virus Infection in Mice. II. The Effect of Penicillin and Sulfadiazine on the Developing Lesion. CLAYTON G. LOOSLI. Department of Medicine, University of Chicago, Chicago, Ill.

Fatal pulmonary infections can be produced in mice by allowing them to breathe air for one hour in a 60-liter chamber into which is atomized 4 cc. or more of 10^{-4} dilution of mouse lung suspension of mouse pneumonitis virus. Animals die of extensive pulmonary consolidation from ten to 16 days following exposure. Intranasal inoculation (0.05 cc. of virus suspension) kills mice in 48 hours. There is evidence microscopically that the virus vesicle develops extracellularly on the surface of the alveolar walls as well as intracellularly in the bronchial epithelial cells.

Total daily doses for 16 days of 2,000 units of Penicillin G in saline given subcutaneously (500 units per 0.1 cc. dose) immediately before or after airborne inoculation suppressed the development of the lesion but did not sterilize the lungs. Seventy-two hours after discontinuing penicillin, gross lesions were present and large amounts of virus could be obtained from the lungs of the treated animals. Sulfadiazine (8 mgm. per day in four doses, 0.1 cc. subcutaneously) sterilized the lungs after seven days of treatment.

Penicillin G (8,000 units per day in 8 doses, 0.1 cc. subcutaneously) given at three-hour intervals following intranasal inoculation altered greatly the developing virus vesicle as seen microscopically. Sulfadiazine (20 mgm. per day) prevents the development of the virus vesicle.

The above observations on the effect of antibiotics on the mouse pneumonitis virus may be of value in further elucidating the fundamental nature of the viruses of the *psittacosis lymphogranuloma* group. Detailed reports of the above studies will appear later.

Hypersensitivity to Penicillin. J. F. WALDO and JEANNE T. TYSON. Department of Internal Medicine, College of Medicine, University of Utah, and Salt Lake General Hospital, Salt Lake City, Utah.

In certain instances it has been possible to demonstrate by passive transfer, employing the Prausnitz-Kustner technique, a circulating antibody to penicillin. This passive transfer was best accomplished from severely reacting subjects during the most active phase of the reaction. The antigen employed for the passive transfer was crystalline Penicillin G dissolved in saline.

By binding crystalline benzyl penicillin (Penicillin G) to pure human albumin and injecting this in rabbits an antibody has been produced which gives a moderately strong complement fixation reaction with the albumin-penicillin mixture after all reactivity to human albumin has been absorbed. Penicillin alone, administered to the rabbit, has failed to produce such an antibody. Some evidence has also been obtained which indicates that when penicillin is bound to human albumin, the reacting anti-

genic product behaves in the manner of a mixed antigen while the human albumin used in the experiment behaves in the manner of a pure antigen. Quantitative studies of this antigen-antibody system seem to substantiate this.

Comparative studies with n-heptyl penicillin (Penicillin K) are now in progress because it is believed by most observers that this penicillin is more completely bound to the protein than is benzyl penicillin. If this is true the antibody reaction should be more marked.

Inasmuch as it is known that penicillin is bound *in vivo* to albumin it seems reasonable to suppose that when penicillin is administered to man, the penicillin haptene coupled to albumin *in vivo* constitutes an antigen which leads to the production of an antibody specific for this haptene group. This might well account for the penicillin sensitivity reactions observed in man.

The Jarisch-Herxheimer Reaction in Early Congenital Syphilis. A Study of 93 Patients Treated with Penicillin Alone. HAROLD A. TUCKER¹ and OSVALDO A. PARDO.² U. S. Public Health Service and the Johns Hopkins Venereal Disease Research and Post-Graduate Training Center, Baltimore, Md.

Between 1943 and March, 1948, 93 children under three years of age were diagnosed as having untreated early congenital syphilis at the Johns Hopkins Hospital. Each was treated with penicillin as the sole antisyphilitic agent. A fever of 38° C. or greater in a previously afebrile patient, occurring from four to 36 hours after the start of penicillin administration and lasting less than 36 hours, was interpreted as a febrile Jarisch-Herxheimer reaction. Forty-five children (48.5 per cent) showed such reactions.

No relationship between incidence and magnitude of the reaction and age, race, sex, type of congenital syphilis, serologic titer or outcome, was observed. The type of penicillin (e.g., amorphous or crystalline G) likewise did not appear to be a factor nor, within the range of 250 to 7,690 units/kg. of body weight, did the gravimetric dosage given. When it occurred, the reaction seemed to be entirely analogous to that seen in the adult with early acquired syphilis.

Five of the 93 children died and in each case prematurity, malnutrition, general debility and/or intercurrent disease were complicating factors. None of these deaths was believed by us to be directly attributable to the Jarisch-Herxheimer reaction. It was our feeling that the outcome was largely dependent upon the general condition of the patient, and that a successful result depended as much, or more, on general pediatric management as upon the type or amount of syphilotherapy employed.

Treatment of Various Infections with a Single Injection or Injections at Five Day Intervals of 300,000 Units (1 cc.) Procaine Penicillin in Oil with 2 per cent W/V Aluminum Monostearate. WALTER KURLAND and HAR-

OLD L. HIRSH. Georgetown University Medical Division, Gallinger Municipal Hospital, and the Department of Medicine, Georgetown University School of Medicine, Washington, D. C.

The present study was undertaken to determine whether common mild and moderate infections can be adequately treated by a single 1 cc. dose of procaine penicillin in oil with aluminum monostearate containing 300,000 units.

Included in this series were 41 patients with scarlet fever. In all but three, throat cultures became negative within 48 hours after administration of the penicillin. These three became negative after 72 hours. One of the three became positive again after 96 hours and persisted as a carrier until a second dose was given on the 11th day, whereupon he again became negative. Two of the 41 patients developed complications. One developed a serous meningitis on the seventh day which cleared rapidly within several days. Another patient developed a purulent otitis media on the tenth day, after having been taken home inadvertently on the preceding day by her parents. Temperatures fell to within normal limits within 48 to 72 hours in all patients except three, and in two additional patients therapy was given. It was strongly suspected that one of these patients did not receive his initial dose and the other required sulfonamides.

Of the 82 patients with lobar pneumonia, three failed to respond adequately to the single injection but recovered following the administration of additional doses, and in two suppuration in the pneumonic area developed which was treated with intensive antibiotic and chemotherapeutic drugs. Seven patients, all with serious complications, died: delirium tremens (2); severe cardiac disease (2); diabetic acidosis (1); asthma with purulent bronchitis and an acute lung abscess at another site (1); and possible renal disease (1).

Successful results were obtained in nine patients with otitis media; one with a hemolytic streptococcus pharyngitis; one with a Vincent's infection of the throat, and three with cellulitis.

Delayed Administration of Oral Penicillin¹ as Prophylaxis for Gonorrhea. V. W. H. CAMPBELL,² WILLIAM J. DOUGHERTY,² and C. E. CURTIS.³ Communicable Disease Control Section, Department of the Navy, Washington, D. C.

The study of the prevention of gonorrhea by Eagle *et al.* has indicated that the utilization of per oral penicillin, 100,000 to 250,000 units, within a short time after venereal exposure is highly effective in the prevention of gonorrhea.

The operation of a Naval Force in an area where high venereal disease rates prevailed afforded the opportunity to conduct further study designed to determine the feasibility and efficiency of prophylaxis by oral penicillin given 12 to 24 hours after exposure to venereal disease.

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³ The penicillin tablets and placebos used in this study were furnished by Commercial Solvents Corporation.

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Two thousand and eighty men participated in the study. One thousand and fifty-nine men in the experimental group experienced 1,833 liberties and 2,625 exposures, resulting in nine cases. This yielded a rate of 4.75 cases per 1,000 liberties and 3.4 cases per 1,000 exposures.

One thousand and twenty-one men in the control group experienced 1,891 liberties and 2,686 exposures, resulting in 22 cases of gonorrhea. This yielded a rate of 11.6 cases per 1,000 liberties and 8.2 per 1,000 exposures.

The experimental group received 200,000 units of sodium Penicillin G by mouth following a mean elapsed interval between exposure and penicillin prophylaxis of 15 hours. The variation about this mean was approximately 3.5 hours.

Contrasted to the study reported by Eagle *et al.*, in which the probable elapsed time of two hours between exposure and per oral penicillin was reported, this study indicates that within an increasing interval between exposure and utilization of the tablets, the prophylaxis efficiency of per oral penicillin is reduced.

Use of Penicillin in Oral Vincent's Infection as a Means of Rapidly Controlling "Epidemics." J. LOOBY¹ and CARL A. SCHLACK.² Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.

This project (X-345) was approved by the Chief of the Bureau of Medicine and Surgery, U. S. Navy, 15 March 1944. Oral color photographs were used as controls and, in all, 107 patients were treated. Fifty-two patients were given 25,000 units of penicillin intramuscularly every three hours until 100,000 to 200,000 units had been injected. Fifty-five patients received topical application of 250 units per cc. saline solution; 10 cc. sprayed on tissue and 3 cc. swabbed on lesions (swab remained in mouth for 10-20 minutes). No other form of treatment was employed (no scaling, mouth washes, oral hygiene, change of habits [dietary or otherwise]). Of those receiving intramuscular injections, 50 showed disappearance of painful symptoms and improvement in tissue appearance in 24 to 48 hours; one reached this stage in 72 hours, and one was indeterminate. Of the groups receiving topical application, 44 showed improvement in 24 to 72 hours, nine were indeterminate, and two failed to improve. The majority in both groups showed reduction in bacteriologic smear positives for Vincent's spirochetes and fusiform bacilli after treatment.

It appeared that the intramuscular injection of penicillin was more effective than the topical application as a means of rapidly controlling "epidemics" of oral fusospirochetosis (Vincent's infection).

Further Observations on Protection against the Lethal Effects of Bacterial Endotoxin by Penicillin and its Impurities. WALTER D. HAWK, WILLIAM H. ANDERSON, and C. PHILLIP MILLET with the technical assist-

ance of BARBARA BOWDEN. Department of Medicine, University of Chicago, Chicago, Ill.

Previous findings regarding protection of mice against the lethal effect of certain bacterial toxins by the intraperitoneal administration of penicillin and its impurities have been enlarged and several related questions have been answered.

1. This means of protection, which has proved effective against the endotoxins of meningococcus, gonococcus, *Salmonella aertrycke*, and a number of other Gram-negative organisms, has been shown to be without effect against exotoxins obtained from *Staphylococcus*, *Cl. botulinum* and *Cl. tetani*.
2. Administration of a number of drugs hitherto untested (streptomycin, aureomycin, the anti-histaminics, etc.) has provided none of the protection shown with penicillin.
3. Preliminary administration of sublethal doses of homologous endotoxin (as described by Cantoni) provides only slight protection as compared with that obtained with impure penicillin. Colchicine with penicillin provides no protection.
4. The administration of adrenal cortical extract with penicillin or adrenal cortico-trophic hormone with penicillin provides only slight protection.
5. The intravenous administration of penicillin and its impurities is of some benefit but cannot be carried out satisfactorily because of the toxicity of the drug in its present unpurified state.
6. Several new means of preparing the "penicillin impurities" have been tested. It has been shown that the protective factor(s) in this material is readily obtained by extraction of the "spent beer" discarded after commercial extraction of penicillin from the fermented liquor.
7. A rapid and economical method of bio-assay of the protective factor has been developed.
8. The "penicillin impurities" used in this work have been shown regularly to contain large amounts of the "enhancement factor" described by Welch, Hobby and others. Unusual prolongation of penicillin blood levels following the administration of these materials could not be demonstrated in our experiments.

Certain Effects of Antibiotics on Animal Tissue Homogenates and Subcellular Elements. KENT WRIGHT and DEAN BURK. National Cancer Institute, Bethesda, Md.

These studies were carried out using aerobic methylene blue reduction and Warburg techniques. For the methylene blue reduction studies, mouse livers were homogenized with a mortar and pestle in 0.01 M phosphate. The substrates were malate, pyruvate, acetate, lactate, alcohol, oxalacetate, fumarate, and citrate at 0.01 M and in each case 4 ppm methylene blue. Purified penicillin, a yellow component of crude penicillin, increased the reduction time 100 per cent above that of the control. With malate present, the effect was even more striking. Penicillin and streptomycin increased the reduction time as much as 50 per cent in concentrations of 1,000 ppm or higher. The

¹ Commander, DC, USNR (inactive).

² Commander, DC, USN.

same effects were observed with acetate and in the case of streptomycin also with ethyl alcohol. For pyruvate none of the antibiotics inhibited methylene blue reduction. Other antibiotics studied were subtilin and siderophilin.

The tumors used were the S-91a grown on C mice and the C₃HBA on C₃H mice. The time of reduction of methylene blue by the S-91a tumor fractions was one-fifth the time required by liver homogenates. This was further affected by penichromin (> 1,000 ppm) to the extent of 100 per cent.

The particulate fractions were prepared by homogenizing the tumors in 0.85 per cent saline, filtering through glass wool, and centrifuging at 26g for separation of the nuclei followed by 9,000g for the mitochondria.

In the case of the S-91a tumor homogenates, the manometric studies showed inhibition of succinate oxidation by penichromin at 500 ppm of 50 per cent and no effect by actidione, aspergillie acid, or subtilin.

For the mitochondrial fraction using the C₃HBA tumor in the presence of cobalt at 50 and 20 ppm, the Q_{o2} of 4.6 was decreased to 1.4 and 3.1 respectively. Similar results were obtained with penichromin.

Thus some antibiotics have a striking effect on the enzymes of animal tissues at the subcellular level while some do not except at very high concentrations.

Studies on Bromine-Oxidizable Sulfur-Containing Compounds in Mold Metabolism. CLAUDE H. PLUMLEE and ARTHUR L. POLLARD. Department of Bacteriology, The University of Tennessee, Knoxville, Tenn.

In the course of a quantitative study of the sulfur metabolism of *Penicillium chrysogenum* Q-176 it was found that sulfur compounds oxidizable to sulfate by bromine water were formed and that they accounted for over one-third of the organic sulfur in the culture filtrate. The organism was grown on Czapek-Dox medium containing sulfur only in the form of magnesium sulfate. It was certain, therefore, that all forms of organic sulfur were synthesized by the organism. Other investigators have found that the sulfur in thiamine, biotin, cysteine, cystine, and glutathione is not oxidizable to sulfate by bromine water. Apparently this oxidation is confined to very specific configurations of the molecule such as are found in the xanthogenic acids and in thioacetamide. It is not clear whether such compounds are intermediates in the process of penicillin synthesis or whether they are formed in equilibrium with other sulfur compounds, one of which is penicillin.

The subject is of considerable interest because of the great number of physiologically active sulfur compounds and the wide variation of activity with molecular configuration. Thioacetamide, for example, has been shown to be readily utilized by *Aspergillus niger* whereas some of the xanthates, thiuram derivatives and disulfids are highly fungistatic.

Streptomycin

The Disappearance of Streptomycin-Resistant Bacteria During or After Streptomycin Therapy. MORTON HAX-

BURGER and JEROME R. BERMAN. Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio.

Though the appearance of streptomycin-resistant bacteria during the course of streptomycin therapy has been abundantly demonstrated, less attention has been paid to the ultimate fate of these resistant forms. Observations upon this problem have been made during a study of the development of resistant coliform bacilli in the stools of patients given intramuscular streptomycin for tuberculosis or oral streptomycin for intestinal tract infections.

Suspensions of 0.5 cc. stool in 5 cc. saline were streaked on each of two eosin-methylene blue plates, one containing 100 gamma streptomycin per cc. and the other no streptomycin. Colonies were picked from representative plates for more accurate determination of streptomycin sensitivity in a serial dilution test.

In no case were coliform bacilli recovered prior to the administration of streptomycin resistant to more than 2.5 gamma per cc. When streptomycin-resistant forms did appear, 156 to more than 2,500 gamma were necessary for growth inhibition.

In five cases (three treated orally and two intramuscularly) where streptomycin was discontinued after the appearance of highly resistant variants, the resistant strains were replaced by sensitive forms. This process started very soon after the cessation of treatment. Whereas during treatment resistant forms were recovered from both the eosin-methylene blue plate containing streptomycin and the plate which contained none, these forms practically disappeared from the latter after treatment. Days or weeks later, even the plate containing streptomycin failed to show growth when stool suspensions were streaked upon it.

In two cases of tuberculous peritonitis where improvement occurred during treatment, the resistant variants disappeared while the patients were still receiving streptomycin.

Evaluation of Oral Streptomycin in Specific Enteritis in Infants and Children. SIDNEY ROSS, E. CLARENCE RICE, FREDERIC G. BURKE, and JOHN A. WASHINGTON. Children's Hospital, Washington, D. C.

Thirty-four cases of shigella enteritis were treated with oral streptomycin. Twenty-five of the cases were acute while nine were carriers. The average dose of streptomycin orally was 400 mgms. every four hours. The duration of treatment ranged from seven to 19 days with an average of 10.7 days per patient. In the acute cases the positive stool cultures became negative rapidly generally within an average of one to two days. In the carrier group none of the 12 cases showed positive stool cultures within 24 hours following initiation of therapy. An average of 11 negative stool cultures was obtained on each patient before he was considered cured. Once the stool culture became negative, no cultural reversals were noted during the hospital stay. In five of the cases, however, there was a recurrence of positive stool cultures within one month after discharge from the hospital.

Clinically, the improvement was commensurate with the salutary effect observed bacteriologically.

Eight cases of salmonella enteritis in infants and children were treated with streptomycin in large doses ranging from 2.4 to 3.6 gms. per day. Three of the patients received the drug orally only while the other five were given the antibiotic both orally and intramuscularly. In spite of the demonstrated sensitivity of the organism to streptomycin *in vitro* in each case, only one of the eight cases was rendered permanently free of the organisms. In the other seven cases an inhibitory effect on both the salmonella organism and the normal stool flora was observed during the period of drug administration; however, within three to eight days after streptomycin was discontinued, the pathogen reappeared in the stools. No significant increase of streptomycin resistance during the course of therapy was noted in any case.

The Treatment of Tuberculosis with Small Doses of Streptomycin. BERNARD MILLOFF, SOL KATZ, and HAROLD L. HIRSH. Georgetown Medical and Tuberculosis Divisions, Gallinger Municipal Hospital, and the Department of Medicine, Georgetown University School of Medicine, Washington, D. C.

Although the value of streptomycin in the treatment of tuberculosis has been rather fully established, a complete appraisal of dosage and duration of treatment has not been ascertained. Therefore, we have treated a variety of tuberculous infections with small doses of streptomycin. All patients received one-half gram per day in one dose except four with miliary tuberculosis who were given one-half gram twice a day. This dose schedule was selected on the supposition that with the smaller doses the development of organism resistance is not increased and streptomycin toxicity is decreased.

The following results were obtained:

Miliary—5—all developed meningitis and died;
Bronchial—4—favorable response after prolonged therapy;
Pericarditis—2—one favorable response, one failure;
Pleurisy with effusion—1—no response;
Peritonitis—2—relapse after improvement;
Mediastinitis—1—favorable response;
Adenitis—3—no response;
Osseous—4—no response;
Draining sinuses—7—apparently healed.

In two patients with tuberculous bursitis incision and drainings while under streptomycin therapy resulted in healing.

In forty-two patients with 80 operations, which included thoracoplasty, lobectomy and pneumonectomy, no tuberculous spreads occurred with the use of one-half gram per day in a single injection for seven days pre-operatively and post-operatively.

Streptomycin in doses of one-half gram per day would appear to be definitely effective in the treatment of bronchial tuberculosis and draining sinuses. It would also be recommended that this same dosage be used in the prevention of tuberculous spreads in the surgery of tuber-

culous patients. In those tuberculous infections where streptomycin is generally effective, smaller doses are apparently as good.

Incidence of Vestibular Dysfunction Following Intramuscular Administration of Various Doses of Dihydrostreptomycin. SOL KATZ, WALTER KURLAND, and HAROLD L. HIRSH. Georgetown Medical and Tuberculosis Divisions, Gallinger Municipal Hospital, and the Department of Medicine, Georgetown University School of Medicine, Washington, D. C.

Recently a reduced streptomycin compound, dihydrostreptomycin, has been developed which appears to be less neurotoxic in preliminary studies in animals and humans.

On the basis of these data a study of dihydrostreptomycin was undertaken. Included in our series are 26 patients. Five patients received 1 gram per day in divided doses every six hours and none have developed evidences of vestibular dysfunction after 13 weeks of therapy. However, of three patients on 0.5 gram every six hours (2 grams per day) for 13 weeks one showed vestibular dysfunction after the sixth week of therapy and the other after the ninth week.

Doses of 3 grams per day (0.75 gram every six hours) were administered in eight patients. Three were treated for 13 weeks and one for 60 days without evidences of impairment of vestibular function. Two demonstrated impairment after six and eight weeks of treatment, respectively. In one, vestibular dysfunction appeared after the first treatment week and in another, a 13 year old boy, after the seventh week.

Three patients were given 3 grams per day in doses of 1.5 grams every 12 hours for a period of four to five weeks. Two developed vestibular impairment after the first and second weeks of therapy, respectively. One was 69 years of age and the other had unilateral dysfunction from a previous course of 150 grams streptomycin. One patient received 1 gram of dihydrostreptomycin for 14 days followed by 3 grams for 18 days. He showed no impairment of vestibular function.

Five patients received 1 gram every six hours for 7, 35, 35, 60 and 60 days, respectively, without impairment of vestibular function. Vestibular dysfunction was noted in one patient on this dosage-schedule after the fourth week of therapy.

The Therapeutic Use of Antibiotics: Comparative Activities, Synergism, and Resistance

The Summation of Penicillin and Streptomycin Activity In Vitro and in the Treatment of Subacute Bacterial Endocarditis. WILLIAM C. ROBBINS and RALPH TOWNSETT. Department of Medicine of the New York Hospital-Cornell University Medical College, New York, N. Y.

Despite the fact that the summation of action of two drugs is an established principle of pharmacology, few examples of unquestionable summation of the action of

two antimicrobial agents in the treatment of infection in man are to be found.

The therapeutic effectiveness of penicillin in subacute bacterial endocarditis has resulted in the arrest of the infection in all but 10 per cent of cases. The majority of this latter group of patients are infected with *Streptococci* of the Lancefield serologic Group D, the most important members of which are commonly termed *Streptococcus fecalis* and *Streptococcus zymogenes*. These enterococci are highly insensitive to penicillin, and although temporary suppression of the endocarditis caused by these organisms is readily attained with its administration, relapse almost invariably ensues after the penicillin is discontinued. Enterococci also exhibit considerable resistance to streptomycin, and results of the treatment of enterococcal endocarditis with streptomycin, though of limited extent, have been only slightly better than those achieved with penicillin.

A study has been made of the individual and combined actions of penicillin and streptomycin *in vitro* on strains of *Enterococcus* and *Streptococcus viridans* isolated from patients with endocarditis. It has been observed that the two drugs act as adjuvants in inhibiting growth of all strains studied, total summation of partial effects occurring throughout a wide range of concentrations of each constituent drug. For example, complete inhibition of growth was achieved with a mixture containing one-fourth the inhibitory concentration of penicillin plus three-fourths of the inhibitory concentration of streptomycin, or with a mixture containing one-half the inhibitory concentration of each. In some instances inhibition has occurred with constituent drug fractions which total less than one, indicating an effect greater than summation.

Six patients with persistent enterococcal bacteremia, five of whom had endocarditis, have received six million units of penicillin and 2 grams of streptomycin, or its dihydro derivative, daily. Two patients received the combined therapy only terminally, but four completed a course of four to eight weeks of this therapy. Bacteremia was promptly reversed and there was sustained clinical improvement in all four. All but one patient have been followed for periods of from six to 12 months and none has relapsed.

The uniformly good results with these patients have been in striking contrast with the previous experience with the use of either drug alone in these infections, and suggest the operation of a summation effective *in vivo* comparable to that observed *in vitro*. Experiments are now in progress which indicate that the summation of penicillin and streptomycin may apply to organisms other than penicillin-resistant streptococci, and that summation may occur with other antibiotic combinations. The use of combinations of antimicrobial drugs may offer an effective method of achieving permanent arrest of infections which can now only be suppressed by the use of one drug alone.

Pharmacological Studies on Actidione. ANNESS GOTH and ELEANOR J. BLISS. Department of Physiology

and Pharmacology, Southwestern Medical College, Dallas, Texas.

Actidione is characterized by a marked growth inhibitory activity against certain fungi and by a considerable species variation in its toxicity to mammals. The purpose of the present study was twofold: to demonstrate inhibitory blood levels in various species of mammals following the injection of actidione and to study the nature of its toxicity.

A solution of crystalline actidione was injected intraperitoneally into rats, guinea pigs, and dogs. The citrated plasma of these animals was tested by a serial dilution method in tryptosephosphate broth for inhibitory activity against *Saccharomyces pastorianus* ATCC2366.

After the injection of 1 to 10 mg. of actidione per kg. into rats, 5 to 20 dilution units were detected in the plasma in 30 minutes. The inhibitory activity decreased somewhat in 90 minutes. The results were essentially the same in guinea pigs and dogs.

It appears from these data that actidione is absorbed rapidly from the intraperitoneal site and is not rapidly inactivated in the body.

The injection of as little as 1 mg. of actidione per kg. body weight intravenously into the dog is followed by vomiting in a few minutes. This emetic effect is prevented by nembutal anesthesia. Nausea has also been observed in humans following the injection of actidione.

Comparisons of Six New Antibiotic Agents in Experimental Infections in Mice. ELEANOR A. BLISS and PATRICIA TODD. Department of Preventive Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md.

The therapeutic activities of penicillin O, aureomycin, chloramphenicol, polymyxin D, polymyxin B and Q 19 (circulin) were compared in mouse infections induced with a type I pneumococcus, a Group A hemolytic streptococcus, and a strain of *K. pneumoniae*, type A. Penicillin G and streptomycin were included as standards. Two strains of mice were employed, a mixed strain of Swiss mouse and the pure CF₁ strain. Therapy was administered by the subcutaneous route, three treatments being given at 0, 5½, and 23 hours after infection. Two or more agents were compared in each experiment, usually at three doses each. The median protective dose was estimated.

The polymyxins and Q 19 were most effective in the therapy of the *K. pneumoniae* infection; the PD₅₀ for all three was less than 1 mg/kilo per treatment. The PD₅₀ for streptomycin was 2.5 mg/kilo; for aureomycin and chloramphenicol it was 35 to 50 mg/kilo. In the treatment of the streptococcal infection, Penicillin G and O were most effective with PD₅₀'s of about 0.3 mg/kilo. The PD₅₀ of aureomycin was 4 mg/kilo while that of streptomycin was 70. The PD₅₀ for chloramphenicol here and in the pneumococcal infection could not be determined because of the limited solubility of the agent, even in 20 per cent alcohol. It was above 80 mg/kilo. In the pneumococcal infection the same order of activity pre-

ailed, with the notable exception that aureomycin was the most effective agent in the CF₁ mice. The PD₅₀ for Penicillin G in this mouse was ten times as high as in the mixed strain of mice.

The Effect of Antibiotics on the Growth of Histoplasma Capsulatum in Vitro. BEN D. CHINN and MARIO MOL-LARI. Department of Bacteriology and Preventive Medicine, Georgetown University School of Medicine, Washington, D. C.

A study was made of the effect of several antibiotics on the growth of *Histoplasma capsulatum* in vitro. *H. capsulatum* is the etiological agent of Histoplasmosis, a disease characterized by emaciation, leukopenia, anemia and irregular pyrexia, and produces cutaneous ulcers and primary involvement of the lungs. The antibiotics used included penicillin, streptomycin, bacitracin, aureomycin, polymyxin A, aerosporin (polymyxin B) and chloromycetin.

The measure of activity against the test organism was determined by the streak method, the agar diffusion method, and the serial dilution method. Penicillin showed inhibitory effects in concentrations above 5,000 units/ml of medium (blood agar). A more marked effect was produced by streptomycin. Inhibition of growth began with 50 mg/ml of medium and only slight growth occurred beyond 200 mg/ml. Chloromycetin showed no antibiotic activity. Aureomycin and polymyxin A showed slight activity above concentrations of 20 mg/ml, but increases in the concentration beyond this amount did not produce a corresponding effect on the growth of the test organism. Bacitracin appeared to have a marked inhibitory effect on the growth of the organism in concentrations of 600 units or higher. Aerosporin had an activity similar to bacitracin, its antibiotic activity beginning with 5 mg/ml.

Among the antibiotics tested streptomycin appeared to have the greatest inhibitory action on *H. capsulatum*. The effect of some of these antibiotics in experimentally infected animals is under investigation.

The treatment of certain forms of Histoplasmosis has been very unsatisfactory; the disease is generally considered highly fatal. The possibility of antibiotic therapy appears worthy of consideration.

The Combined Action of Antibiotics and Sulfonamides in Experimental Infections. C. W. PRICE, W. A. RANDALL, and H. WELCH. Federal Security Agency, Food and Drug Administration, Washington, D. C.

Various combinations of penicillin, streptomycin, aureomycin, chloramphenicol, bacitracin, polymyxin, sulfanilamide, sulfadiazine, sulfathiazole and sulfamerazine were tested in vivo for possible synergistic action, using *Streptococcus hemolyticus* C 203 Mv as a test organism. In these experiments organisms were injected with and without mucin. When mucin was used the virulence of the streptococci was markedly increased; relatively larger doses of the antibacterial agents were required and changes in the ratios of combinations showing a synergistic effect resulted. The results obtained in experi-

mental infection emphasize the difficulty of translating experimental observations into terms of clinical effectiveness.

Alteration and Inhibition of Sea-Urchin Egg Division by Antibiotics. IVOR CORNMAN. Sloan-Kettering Institute for Cancer Research, New York, N. Y.

As a part of a cancer chemotherapy screening program, sea-urchin eggs are being used to detect substances which inhibit cell division and in the study of their effects on mitosis. The eggs of *Arbacia* and *Lytechinus* are exposed ten minutes after fertilization. Normally, cleavage follows at 50-60 minutes after fertilization at 23°-26° C. Divided eggs are counted at intervals to determine inhibition or retardation of cleavage, and such abnormalities as are visible in the living egg are recorded.

Crude penicillin has received special attention because of its ability selectively to kill tumor cells and because Burk has found it to contain a potent metabolic inhibitor. Older pharmaceutical grades of penicillin retarded cleavage at about 300 mg/L. A series of five penicillins supplied by Burk proved to have relative potencies of 1:2:200 (three having the middle rating) compared with respiratory inhibition ratings of 1:10:300. Burk's partially purified *Penichromin* was effective at 5 mg/L, while purified penicillin required 1 g/L to retard cleavage.

A series of filtrates from *Aspergillus* cultured under a variety of conditions were nearly all effective in retarding cleavage. Gliotoxin displayed a distinctly different pattern of action, quickly destroying the eggs when used at concentrations sufficient to delay cleavage (2 mg/L). Percentages of eggs completing second cleavage were: 100 per cent, 30 per cent, and 0 per cent at 1, 2, and 4 mg/L.

Clavacin is another of the more active purified antibiotics, inhibiting all cleavage at 5 mg/L. It was, therefore, more active than its component parts tested separately: the pyrone ring and the five-membered lactone ring.

Search for New Antibiotics

The Isolation and Purification of Neomycin. E. AUGUSTUS SWART, SELMAN A. WAESMAN, and DOVVIS HUTCHISON. New Jersey Agricultural Experiment Station, Department of Microbiology, Rutgers University, New Brunswick, N. J.

Neomycin has been isolated from culture filtrates of *Streptomyces* 3535 by the following sequence of steps: acidification to pH 2.0-2.5, clarification with activated charcoal, neutralization, adsorption on decalin, elution with 10 per cent ammonium chloride, adsorption on activated charcoal from pooled ammonium chloride eluates at pH 7.0, elution by 0.05 N hydrochloric acid in 50 per cent methanol, neutralization by passage through an amberlite IR-4B column or by addition of aqueous sodium hydroxide, concentration "in vacuo" at 35-35° C. to 0.01-0.1 of original volume, picking up residue in methanol and precipitating with acetone, or picking up residue in water and lyophilizing. The concentration of neomycin

chloride showed an average potency of 100 units/mg, some solids ranging as high as 225 u/mg.

Chromatography on columns of Darco G-60 or alumina, using methanol as solvent and eluant, has yielded more highly potent concentrates. Precipitation with picric acid, followed by direct conversion to the hydrochloride, has also given more potent solids.

Another antibiotic, having the same antibacterial spectrum as neomycin but having antifungal activity as well, has been isolated from the culture filtrate. Most of this material was removed by the preliminary acidification of the broth and by treatment with Norit H15, leaving neomycin in the filtrate. Norit A, Amberlite IR-100-H, and decalco adsorbed neomycin from culture filtrate, leaving the second agent in the filtrate.

Neomycin is soluble in water, slightly soluble in methanol, and insoluble in other organic solvents. It is stable at pH 2.1 for one hour at 100° C, four hours at 25° C, and five days at 5° C. A negative Sakaguchi test indicates the absence of mono-substituted guanidine groups. The maximum in the distribution curve occurs at tube 21 when neomycin is distributed between pentasol and borate buffer at pH 7.6, using stearic acid as a carrier, in the 24 plate Craig Counter-current Distribution Machine. Streptomycin and streptothricin show respective peaks, in the same system, at tubes 9 and 8.

Borrelidin, a New Antibiotic with High Anti-Borrelia Activity and Penicillin Enhancement Properties, Produced by a Streptomyces Species. J. BERGER and M. W. GOLDBERG. Research Laboratories of Hoffmann-La Roche, Inc., Nutley, N. J.

An antibiotic with unusual specificity and high activity has been found to be produced by several actinomycetes. It is highly active *in vitro* against *Sarcina lutea* and certain micrococci but relatively inactive against other common test bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. The growth of a protozoan, *Tetrahymena geleii*, is inhibited at a dilution of 1:200,000. As little as 0.0001 mg. per ml. can be detected by the cylinder plate technique when *S. lutea* is used as test organism. The substance has been isolated in crystalline form (L. M. Jampolsky and M. W. Goldberg) and was named "Borrelidin" because of its outstanding activity *in vivo* against *Borrelia* species (R. J. Schnitzer *et al.*).

The culture producing the best yield of borrelidin is a new *Streptomyces* species. It grows readily on complex organic media, but only sparingly on synthetic liquid media. Under usual methods of handling, the culture yields strains which vary widely in appearance and antibiotic production. Borrelidin is produced by the *Streptomyces* in a variety of liquid media, in stationary shallow layers or in aerated submerged culture. Particularly suitable are media containing soybean meal, glucose, and phosphate.

Small amounts of borrelidin-like substances have been found in sources of penicillin enhancement factors, such as inactivated impure penicillin, clarase enzyme prepara-

tions and even in corn steep liquor. In spite of the fact that pure borrelidin does enhance penicillin activity *in vivo* (E. Grunberg *et al.*), no claim can be made for its identity with any of the postulated penicillin enhancement factors since certain differences have also been observed.

Isolation of Crystalline Borrelidin from Streptomyces Broth and of Borrelidin-Like Concentrates from Crude Penicillin. L. M. JAMPOLSKY and M. W. GOLDBERG. Research Laboratories of Hoffmann-La Roche, Inc., Nutley, N. J.

A new antibiotic, borrelidin, has been isolated in crystalline form from the culture medium of a new *Streptomyces* species. The antibiotic exhibits an unusual degree of specificity, being highly active only against *Sarcina lutea in vitro* and against several strains of *Borrelia in vivo*. Concentrates exhibiting some of the biological properties of borrelidin were also obtained from crude penicillin and other sources reported to contain penicillin enhancement factors. Borrelidin itself was found to enhance the activity of penicillin in certain experimental infections of mice (E. Grunberg *et al.*).

Borrelidin can be isolated from the culture solution by extraction with butyl acetate. The residue obtained by evaporation of the extract can be purified by treatment with bentonite clay in various solvents and finally, by extraction from ether with alkali. Concentration of a benzene extract of the acidified alkali solution yields crystalline borrelidin. Recrystallization from benzene gives pure borrelidin, m.p. 145-146°, $[\alpha]_D^{25} = -28^\circ$ in ethanol.

Borrelidin is an acid. A crystalline methyl ester (m.p. 153-154°) and a crystalline p-nitrobenzyl ester (m.p. 161°) have been obtained. The methyl ester yields a crystalline diacetyl- (m.p. 190°) and a crystalline di-p-nitrobenzoyl-derivative (m.p. 156-157°). The micro-analytical data so far obtained indicate an empirical formula of $C_{22}H_{44}O_6N$ for borrelidin. The ultra-violet absorption spectrum in isopropanol shows a maximum at 256 $m\mu$ ($E_{1\%}^{1\text{cm}} = 550$), indicating a site of conjugated unsaturation in its molecule.

Enhancement of Penicillin Activity by Borrelidin. E. GRUNBERG, D. ELDRIDGE, and G. SOO-HOO. Research Laboratories of Hoffmann-La Roche, Inc., Nutley, N. J.

The new antibiotic, borrelidin, the principal chemical and biological properties of which have been described in the preceding abstracts, did not show any demonstrable chemotherapeutic activity *in vivo* if tested in experimental infections with β -hemolytic streptococci, pneumococci type 1 and 2, *Klebsiella pneumoniae*, *Salmonella schottmuelleri*, *Eberthella typhosa*, *Mycobacterium tuberculosis*, strain H37Rv, and *Cryptococcus hominis*. This substance, however, possessed some growth inhibiting effect *in vitro* against the organisms enumerated above.

Attempts were made to show that borrelidin possesses properties of enhancement of crystalline Penicillin G similar to those described for unknown factors obtained by inactivation of impure penicillin (Hobby *et al.*, Dunham

et al., Miller *et al.*, Cole, Burk *et al.*, and Welch *et al.*). It could indeed be demonstrated that the addition of very small amounts of borrelidin increased the effect of crystalline Penicillin G in experimental infections with group A, type 3 hemolytic streptococci. The findings may be summarized by the comparison of the CD50 of Penicillin G in the presence and absence of borrelidin.

Single treatment	CD50 of Penicillin G
Cryst. Penicillin G alone	0.56 mg/kg (935 units/kg) subcut.
Cryst. Penicillin G + borrelidin 0.2-1.7 μ g/kg	0.25 mg/kg (417 units/kg) subcut.
Cryst. Penicillin G alone	1.16 mg/kg (1,937 units/kg) per os
Cryst. Penicillin G + borrelidin 3.0 μ g/kg	0.71 mg/kg (1,185 units/kg) per os

There was also found a marked trend to enhancement of Penicillin G by borrelidin in experimental rabbit syphilis, in the protection of mice against meningococcal endotoxin, and in the growth inhibition of *Trichomonas vaginalis*. In addition borrelidin inhibited the respiration of staphylococci as tested by the methylene blue method.

We were, however, not able to demonstrate with borrelidin the enhancement of Penicillin G in infections with *E. typhosa* as described by Welch and with type 1 pneumococci as described by Hobby. The enhancement of Penicillin G in infections with *Salmonella schottmuelleri* was also not observed.

No definite statement can be made at the present time regarding the identity of borrelidin and any of the enhancement factors described.

The Anti-borrelia Effect of Borrelidin. R. J. SCHNITZER, M. BUCK, and A. C. FARR. Research Laboratories of Hoffmann-La Roche, Inc., Nutley, N. J.

During the routine screening in experimental infections of mice it was found that borrelidin, a new antibiotic found and isolated in crystalline form by M. W. Goldberg and his associates, J. Berger and L. M. Jampolsky (see the abstracts of these authors), exerted a striking effect on borrelia infections. This is illustrated by the following table:

Animal	LD50	CD50: mg/kg		
		Borrelia tick	B. Noryi	B. Obermeieri
Mice	74.7	0.6	2.0	1.7
Rats	1.78	—	—	0.9

Borrelidin-like substances in concentrates from impure penicillin, clarase and corn steep had a similar activity.

Borrelidin was also effective toward a partially penicillin resistant strain of *B. Noryi*. It was comparatively easy to produce a borrelidin fast strain of *B. Obermeieri* which still responded to As-compounds, Au-compounds, penicillin and aureomycin. This indicates that borrelidin represents a type of anti-borrelia agent different from those mentioned before.

No effect of borrelidin was seen in experimental rabbit syphilis and in trypanosomal infections. The compound exerted, however, a growth inhibiting effect on *Trichomonas vaginalis* *in vitro* in concentrations of 0.004-0.006 mg/ml.

Borrelidin is a substance of considerable toxicity. The LD50 for mice was found to be 74.7 mg/kg and 39.0 mg/kg if given by the subcutaneous and intravenous route respectively. Other animals, *e.g.*, rats and rabbits, were considerably more sensitive to this material. Moreover, the new antibiotic is irritant to the tissues of animals and sometimes to the skin of humans.

Candidulin: an Antibiotic from *Aspergillus candidus*. P. G. STANSLY and N. H. ANANENKO. Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Conn.

A substance has been recovered from the fermentation liquor of a strain of *Aspergillus candidus* which has marked activity against acid-fast bacteria. The properties of the active principle distinguish it from known antibiotics. The name "candidulin" is proposed for it.

Moderately active fermentation liquor may be obtained in stationary culture on a neutral substrate consisting of glucose, sodium nitrate and potassium phosphate. The active principle may be removed from the metabolic liquor with chloroform and crystallized from n-hexane. The yield of purified material is about 5 mg. per liter. The substance is a colorless, neutral, non-aromatic compound, m.p. 88-89°, $[\alpha]_D^{25} = +15^\circ$ (chloroform). Elemental analysis is consistent with an empirical formula of $C_{12}H_{12}NO_4$. Treatment with bromine in carbon tetrachloride afforded a biologically inactive crystalline product containing bromine, m.p. 143-147° (dec.).

In broth dilution tests, the antibiotic exhibits moderate activity against some Gram-positive and Gram-negative bacteria. It is particularly effective against the mycobacteria, inhibiting the growth of five strains, representing pathogenic and saprophytic species, in concentrations of approximately 1 μ g. per ml. The substance, however, failed to influence the course of experimental mouse tuberculosis when administered subcutaneously in daily doses of 25 to 200 mg. per kg.

Inhibitory Action of Filtrates from Fungous Cultures on Three Plant Viruses. W. C. PRICE, B. M. GUPTA, and SYLVIA WOLCZEZ. Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pa.

Filtrates from three-weeks-old cultures of about 40 species of fungi grown on lacto-beef-cornmeal-dextrose broth were tested for ability to inhibit infection with Southern bean mosaic, tobacco mosaic, and tobacco ring-spot viruses. Filtrates from *Trichothecium roseum*, *Neurospora sitophila*, and *N. crassa* were found to inhibit infection with all three viruses. The inhibitory action took place immediately whether the filtrate was mixed with the virus or applied to the host before or up to 30 minutes after infection.

¹ Aided by a grant from the National Foundation for Infantile Paralysis.

ter inoculation. Virus preparations rendered inactive by addition of filtrate regained their activity when diluted. These results can be interpreted to mean that the filtrates either render the host insusceptible to the virus or enter into a reversible combination with the virus. Evidence favoring the former interpretation was obtained by study of the reduction in infectivity induced by addition of varying quantities of antiviral agent to a constant dilution of virus. The reduction in infectivity, as expressed in probits (or normal equivalent deviations), was found to be a linear function of the logarithm of dilution.

Marine Organisms as a Source of Physiologically Active Substances. IVOR CORNMANN. Sloan-Kettering Institute for Cancer Research, New York, N. Y.

Every phylum of the animal kingdom has members which possess poisonous tissues or toxic secretions. Representative species have been tested according to the method outlined in an accompanying abstract.

The protozoan, *Gymnodinium brevis*, believed responsible for the "red tide" which periodically kills millions of fish and shellfish, produces a poison which checks the division of sea-urchin eggs.

Sponges crushed in sea water vary in their inhibitory potency. Of ten species tested, two exerted a slight effect only after several hours when diluted 1:100. Four species retarded the first cleavage at 1:100 and showed a delayed effect at 1:1,000. One allowed only 7 per cent of the eggs to divide when the sponge macerate was diluted 1:1,000 and retarded cleavage at 1:10,000. Another produced no measurable effect for several hours and then killed the larvae at all doses tested (1:100, 1:1,000, 1:10,000). These differences in dose-effect relationships reveal a qualitative as well as a quantitative difference in the active agents. The most toxic sponge species permitted only a few eggs to divide and visibly damaged the cytoplasm in a half hour when diluted 1:10,000. Even at 1:100,000 it killed the embryos but required several hours to take effect.

The spines of *Centrechinus* are poisonous enough to produce a painful swelling when they puncture the skin, and many species of fish cannot survive in tanks containing these sea urchins. A brei prepared from the spines proved to be only a weak inhibitor of cleavage.

Numerous mollusks release an ink when disturbed. A large sea slug, *Tethys*, ejects a raspberry-odored red fluid which blocks egg development at 1:1,000. *Thais patula* exudes a white garlic-odored fluid which turns blue. This blue solution retards cleavage when diluted 1:10 and is without influence at 1:100. Mantle fluid from *Thais deltoidea*, which has no ink, is harmless at 1:10.

Aureomycin and Chloramphenicol

The Assay of Aureomycin by Instrumental Methods. HOWARD STONE KETTER and LEON GOLDMAN. Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Two methods based on a standard solution of aureomycin HCl are outlined for measuring the potency of aureomycin solutions.

A fluorometric method involves a five-min. boiling of 100–400 μ g. of the antibiotic with an equimolecular mixture of Na_2PO_4 and Na_2HPO_4 . On dilution, the resulting fluorescence is measured on a Pfaltz and Bauer fluorophotometer, using the same filter system as the Vitamin B₁ assay. A blank solution, made up to the same dilution, employs a PO_4 buffer to give a pH = 10, being read immediately without heating. The difference between readings represents the amount of Aureomycin present.

A colorimetric method uses a sample ranging from 100 to 1,400 μ g. This sample is treated with twice its volume of conc. HCl for 10 min. when it is diluted for reading on a Lumetron Colorimeter (Model 402-E) using M440 filter. A blank solution is prepared using the same ingredients but adding the acid to the diluted sample. The galvanometer is set at 100 per cent transmittance with the blank solution after which the "treated solution" is read.

Both methods give a straight line standard curve on linear paper. Both give results comparing favorably with a microbiological assay method.

*Studies on Assay Methods of Aureomycin in Body Fluids.*¹ C. M. WHITLOCK, JR., A. D. HUNT, JR., and S. G. TASHMAN. Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania, Philadelphia, Pa.

The accuracy of the two basic methods of aureomycin assay which seemed most promising (namely, the serial dilution method of Dornbush and the turbidimetric method of Meads) was assessed by measuring known quantities of aureomycin in the presence of various human body fluids with both methods. Similar data have not been previously reported. For concentrations greater than 0.1 μ g./cc., the serial dilution method was found to be accurate within ± 100 per cent in serum and urine, and to be inaccurate in spinal fluid (errors of 100 per cent or over being common). For similar concentrations, the turbidimetric method was found to be accurate within ± 50 per cent in serum, and ± 100 per cent in urine and spinal fluid.

In the above comparison, the technique used was essentially the same as that of the originators of the methods. When the turbidimetric method was modified by substituting a standard curve containing four points for the single standard used by Meads, the experimental error was reduced to within ± 25 per cent in serum, ± 35 per cent in spinal fluid, and ± 45 per cent in urine. This change also simplifies the method by eliminating most of the mathematical detail work. This laboratory now uses this modified turbidimetric method exclusively for the assay of aureomycin.

¹ These studies were conducted under contract with the Department of the Army, Chemical Corps, Camp Detrick, Md.

Investigation of plate methods of assay was terminated with inconclusive results when it was learned that this type of method measures aureomycin only in concentrations of about 1 $\mu\text{g./cc.}$ or greater.

Using the turbidimetric assay method, it was determined that serum and acid urine specimens can be frozen and assayed in weekly lots without appreciable loss of aureomycin activity. Spinal fluid must be run on the day collected, however, if concentrations of less than 1 $\mu\text{g./cc.}$ are to be accurately measured. When 1 $\mu\text{g./cc.}$ of aureomycin in serum was incubated three hours at 37° C. before assay, there was less than 15 per cent loss of potency. This observation suggests that the degree of destruction of aureomycin under physiological conditions has been over-emphasized.

Aureomycin Studies. I. Effect of Aureomycin on Ten Strains of Virus in the Psittacosis-LGV Group. JOHN C. WAGNER. Biological Department, Chemical Corps, Camp Detrick, Frederick, Md.

A recent report (Cox *et al.*) on the efficacy of aureomycin on the 6BC strain of psittacosis virus made it appear desirable to obtain additional data on the action of this antibiotic on other virus strains within the psittacine group. This report presents the results of such a study in both eggs and mice on ten strains of virus in the psittacosis-lymphogranuloma venereum group.

Drug effect was evaluated in eggs by comparing the LD₅₀ values of each virus strain titrated in untreated as compared to aureomycin treated embryos. In animal studies mice were inoculated intracerebrally with each virus strain and treated, beginning 48 hours later, by five daily subcutaneous inoculations of varying amounts of aureomycin.

In the egg experiments all the virus strains were extremely susceptible to aureomycin. In high dilution the virus was almost completely masked by the presence of drug as manifested by survival of eggs and low titer of virus on subinoculation. As the virus concentration increased, the masking effect could be demonstrated only by survival of treated embryos, but not by titer of recoverable virus within these embryos. All five pathogenic strains (Borg, S-F, Gleason, 6BC, P207) tested in mice were susceptible to the drug. Injections of 0.5 mg. or 0.1 mg. of aureomycin lengthened significantly the life span of treated groups of animals as compared to untreated controls. Living virus was recovered after 21 days from the brains of all treated groups of surviving mice.

The Action of Aureomycin on the Virus of Atypical Pneumonia in Chick Embryos and Cotton Rats. MONROE D. EATON. Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

The virus isolated from patients with atypical pneumonia as described in previous publications¹ is unrelated

antigenically to Q fever or to agents of the psittacosis group, both of which are inhibited by aureomycin. Chick embryos inoculated by the amniotic route with the atypical pneumonia virus received two doses of aureomycin of 1 mg. each into the yolk sac two and 48 hours after the virus was given. The lungs, trachea and amniotic membrane of the treated embryos were pooled, made up in 20 per cent suspension, and inoculated intranasally into cotton rats to test for the presence of virus. Control embryos receiving virus and saline were similarly tested. The cotton rats were sacrificed ten days after inoculation. Of the 27 cotton rats receiving material from the treated eggs, one developed pulmonary lesions, while 20 of the 26 animals receiving control material developed areas of consolidation. When a single dose of 1 mg. of aureomycin was given two hours after the virus, treated eggs produced lesions in eight of 28 cotton rats, and controls in 17 of the 26 cotton rats, a less significant difference probably due to early decomposition of aureomycin in the chick embryo.

Direct treatment of cotton rats infected intranasally with chick embryo material and given daily intraperitoneal doses of 1 to 2 mg. of aureomycin starting 24 hours after inoculation of the virus also resulted in significant therapeutic effects on the pulmonary lesions. The incidence of pulmonary lesions in animals treated in this manner was as follows: 0/9 in those receiving 2 mg. daily for ten days, 1/22 in those receiving 1 mg., and 27/46 in the controls. Preliminary results indicate that the minimal therapeutic dose in cotton rats may be close to 0.5 mg. daily or approximately 10 mg./kg. body weight.

Clinical Evaluation of Aureomycin and Chloramphenicol in Pneumonia in Infants and Children. BENNETT OLSHAKER, SIDNEY ROSS, ADRIAN RECINOS, and ELLSWORTH TWIBLE. Children's Hospital, Washington, D. C.

Aureomycin

Forty cases of pneumonia in infants and children have been treated with aureomycin during the past three months at Children's Hospital. Of these, 32 were pyogenic pneumonias including pneumococcus, streptococcus and staphylococcus. The other eight cases appeared to satisfy the diagnostic criteria of atypical pneumonia.

In the pyogenic group the response both clinically and bacteriologically was striking. The temperature came down precipitously within 12 to 36 hours in almost every case and concomitantly a marked clinical improvement ensued. Roentgenologically, the resolution of the pneumonic process was rapid in the majority of cases; however, in about 20 per cent of the cases there was a lag in the resolution of the area of consolidation by x-ray approximating five to eight days. The drug was well tolerated orally with the exception of the occasional occurrence of nausea, vomiting and diarrhea. Ten of the cases in the infant age group received the drug intramuscularly and showed a prompt response in the majority of instances. However, it was our impression that the intramuscular mode of administration was less satisfactory therapeutically than the oral method of administration.

¹Eaton, M. D., McEldreigh, G., and Van Herick, W. J. *Exp. Med.*, 1945, 82, 317-329. *Am. J. Hygiene*, 1947, 45, 82.

In the eight cases of atypical pneumonia the therapeutic response was similarly gratifying and seemed to follow the favorable course which Schoenbach and Bryer and Finland and his associates have described in their series of virus pneumonias in adults.

Chloramphenicol

Twenty-seven consecutive cases of bacterial pneumonia in infants and children were treated with chloramphenicol. The age range varied from five months to eight years. In 25 of the 27 cases, there was a prompt defervescence of temperature within 12 to 36 hours after initiation of the drug accompanied by marked clinical improvement. The white blood count dropped sharply and the areas of consolidation on x-ray showed resolution within three to six days. The results thus far would indicate that chloramphenicol has definite therapeutic value in bacterial pneumonia.

Some Observations on the Absorption and Excretion of Aureomycin and Chloramphenicol. SIDNEY ROSS, HAROLD BISCHOFF, WARREN PREISSER, and WILLIAM ORR. Children's Hospital, Washington, D. C.

A. Chloramphenicol

- (1) Chloramphenicol was observed to pass through the placental barrier and was present in cord blood in a concentration of 50 to 75 per cent of that observed in the maternal blood.
- (2) Chloramphenicol readily passed the blood-brain barrier and appeared in the spinal fluid in concentrations ranging from 30 to 50 per cent of that noted in the blood stream after oral administration.
- (3) No enhancement in chloramphenicol blood levels was noted after concomitant oral administration of caronamide.
- (4) Chloramphenicol was readily absorbed after rectal administration of 125 to 250 mgms. in infants and appeared in the blood in therapeutic concentrations.

B. Aureomycin

- (1) Aureomycin was found in the pleural and peritoneal fluids of a nephrotic child after oral administration of 250 mgms. every four hours. The concentration of aureomycin in these fluids was approximately one-eighth of that noted in the blood.
- (2) Aureomycin was absorbed into the blood stream after rectal administration of 250 mgms. in children.

Observations on Tuberculous Meningitis Treated with Oral Aureomycin.^{1,2} GEORGE T. HARRELL, MANSON MEADS,³ ROBERT B. LAWSON, and THOMAS N. LIDE.

¹ This study was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

² The aureomycin used in this study was furnished by Lederle Laboratories.

³ Markle Scholar in Medicine.

Departments of Internal Medicine, Pediatrics and Pathology, Bowman Gray School of Medicine of Wake Forest College and the North Carolina Baptist Hospital, Winston-Salem, N. C.

In vitro observations showed that tubercle bacilli were susceptible to aureomycin. This antibiotic was found to penetrate into the spinal fluid after oral administration. These facts suggested possible application of the drug to therapy in tuberculous meningitis.

A four-year-old boy weighing 14 kilo. had fulminating symptoms of meningitis for three days. Acid-fast rods were found in large numbers on direct smear of spinal fluid. A critical study was done on the effect of very large doses of aureomycin (300-500 mg. per kilo., 4-8 grams daily) given only orally over 21 days. Almost daily blood and spinal fluid levels of aureomycin were obtained. Blood levels (2-15 μ g./cc.) varied more widely than spinal fluid levels (1-3 μ g./cc.) and both varied reciprocally with the number of stools (one to eight per day). Organisms disappeared on smear and the spinal fluid sugar rose from 10 to 40 mg.%. Clinical improvement was steady for over two weeks; a sudden worsening in the third week terminated fatally.

At autopsy caseous lesions were found in lymph nodes and spleen with no evidence of healing. The drug apparently did not reach intracellular organisms deep in tubercles. Serial cultures were planted directly and the susceptibility of the organisms to aureomycin was determined after recovery from guinea pigs inoculated with spinal fluid and tissue.

No toxic effects were noted pathologically from this huge dose—the largest yet recorded—administered for a relatively long time. The negative spinal fluid cultures without intrathecal therapy may justify further study of aureomycin in combination with other forms of therapy in this disease.

A Comparative Study on the Effects of Aureomycin and Penicillin on the Pharyngeal Flora of Normal Human Beings.^{1,2} MANSON MEADS,³ WALLACE P. ROWE, and NANCY M. HASLAM. Department of Internal Medicine, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

The effects of aureomycin and penicillin on the aerobic bacterial flora of the pharynx were compared in normal human subjects. Repeated semiquantitative throat cultures were taken from the same individual before, during, and after the successive oral administration of the two antibiotics in different doses. By utilizing each subject as his own control, host factors that may influence chemo-

¹ This investigation was supported (in part) by a research grant from the Bacteriology Study Section, Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

² The aureomycin and penicillin used in this study were furnished by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., through the courtesy of Dr. Stanton M. Hardy.

³ Markle Scholar in Medicine.

therapeutic response were minimized. Repeated tests were made of the *in vitro* susceptibility of the bacteria recovered throughout the period of observation. The *alpha hemolytic streptococci* proved to be a good index for the comparison of certain aspects of the antibacterial activity of the two drugs because of (1) their susceptibility to aureomycin and penicillin, (2) their constant appearance in normal pharyngeal cultures, and (3) their consistent reappearance after effective therapy had been stopped.

With the exception of the genus *Hemophilus*, aureomycin and penicillin demonstrated a similar spectrum of antibacterial activity. By weight, penicillin was approximately five times more effective than aureomycin against *alpha streptococci* in the human pharynx; penicillin was 12 times more active against these organisms *in vitro*. In most instances, a drug-specific decrease in the mean *in vitro* susceptibility of *alpha streptococci* and *Neisseria flava* paralleled a failure to clear these organisms from the pharynx. The mean increase in resistance to penicillin was maintained throughout a three-week period after this drug had been discontinued. The degree of aureomycin-fastness that followed treatment with this agent slowly decreased *in vivo*, although repeated transfer of resistant organisms in media free of antibiotic failed to effect their degree of susceptibility. The apparently stable property, fastness to penicillin, was used as a label to study the dynamics of aureomycin-fastness in pharyngeal bacteria.

This method of antibiotic comparison may serve as a valuable tool to supplement laboratory studies on a new chemotherapeutic agent before it is used in clinical disease.

Isolation and Chemistry of Chloramphenicol (Chloromycetin¹). QUENTIN R. BARTZ. Research Laboratories, Parke, Davis and Company, Detroit, Mich.

The activity of chloramphenicol, a new crystalline antibiotic obtained from filtrates of submerged aerated cultures of *Streptomyces venezuelae*, was followed by means of a turbidimetric method employing *Shigella paradysenteriae* (Sonne) as the test organism. It was stable at 25° in culture filtrates over the pH range of 0.40–9.56 for 24 hours but was rapidly destroyed at pH 10.82. The distribution ratio at 25° of chloramphenicol between organic solvent and either acidified or weakly alkaline aqueous solution was found to be three or more for cyclohexanone, butanol-1, ethyl acetate, methyl isobutyl ketone, isopropyl acetate, amyl acetate, methyl amyl acetate, nitrobenzene, nitromethane and diethyl ether; approximately one for ethylene dichloride, and less than one for chloroform, carbon tetrachloride, trichloroethylene, benzene, dichloroethyl ether, and petroleum ether. Chloramphenicol is readily absorbed by Nuchar C190N from aqueous solution at either an acid or neutral pH.

Two general procedures for the isolation of chloramphenicol in crystalline form from crude culture filtrates involve (1) extraction with water-immiscible organic

solvents or (2) adsorption on carbon and elution with organic solvents.

Chloramphenicol is an optically active, nonionic chlorine-containing amide, melting at 149.7–150.7°, the molecular formula of which is $C_{12}H_{14}ClN_2O_6$. The assigned structure has been confirmed by an unequivocal synthesis (Controulis, Rebstock and Crooks).

Chloramphenicol (Chloromycetin¹) Assay and Susceptibility Test Methods. D. A. JOSLYN, J. EHRLICH, and J. L. SCHWAB.² Research Laboratories, Parke, Davis & Company, Detroit, Mich.

The chloramphenicol content of aqueous solutions and body fluids has been determined by three microbiological methods. *Turbidimetric Method*: Samples are diluted to an estimated concentration of 0.2 microgram chloramphenicol per milliliter in brain heart infusion (Difco) containing a standardized inoculum of *Shigella sonnei*; after *ca.* four hours' incubation at 37° C., potency is calculated by comparing turbidity of the sample tubes with that of control tubes containing 0.1 to 0.5 µg. of a standard chloramphenicol per milliliter. *Overnight Broth Method*: Samples are diluted serially as required in brain heart infusion containing a standardized inoculum of *S. sonnei*; after *ca.* 18 hours' incubation at 37° C., the concentration of chloramphenicol in the sample is estimated by comparing the end point (complete inhibition) of the sample with that of a standard. *Agar Diffusion Method*: Uniform volumes of appropriately diluted samples and standard are added to one-half inch diameter filter paper disks placed on agar plates seeded with *Bacillus subtilis* or other susceptible test organism; potency is estimated by comparing the resulting inhibition zone diameters with aid of a standard curve.

The susceptibility of cultures, particularly those isolated from candidates for chloramphenicol therapy, has been tested by two microbiological methods. *Agar Dilution Method*: Tryptose agar dilutions of chloramphenicol are streaked with clinical material; after suitable incubation, presence or absence of growth in the several plates indicates the degree of susceptibility. *Broth Dilution Method*: Tryptose broth dilutions of chloramphenicol are inoculated with a standardized suspension of the cultures on test; after suitable incubation, the degree of susceptibility is indicated by complete inhibition of growth.

A Colorimetric Method for the Determination of Chloramphenicol (Chloromycetin¹); Application to Biochemical Studies. ANTHONY J. GLAZO, WESLEY A. DILL, and LORETTA M. WOLF. Research Laboratories of Parke, Davis and Company, Detroit, Mich.

The colorimetric method used for the determination of Chloromycetin and related aromatic nitro compounds is based on reduction with metallic zinc or titanous chloride.

¹ The name Chloromycetin has been adopted by Parke, Davis & Company as a trademark for this antibiotic.

² Chloromycetin is the Parke, Davis and Company trade name for chloramphenicol.

³ With technical assistance of M. C. Galbraith, D. Fox, M. L. Davitt, M. F. Sheehan and F. E. Goss.

followed by diazotization and coupling of the resulting aryl amine with the Bratton-Marshall reagent. The active antibiotic can be separated from its metabolic products by extraction with ethyl acetate and determined separately. The titanous procedure has also been used for the development of color on paper strips after chromatographic separation of various Chloromycetin derivatives.

Blood level studies on man and lower animals indicate rapid absorption and excretion of this antibiotic. Human subjects given Chloromycetin by mouth were found to excrete aromatic nitro compounds which accounted for more than 90 per cent of the administered dose in 24-hour urine specimens. Less than 10 per cent of this was found to be active Chloromycetin which was isolated from urine and identified.

The inactive metabolic products were chromatographed on paper strips, and one component was shown to have the same R_f value as a known hydrolysis product of Chloromycetin. The other inactive derivative, representing the major excretory product, was isolated by countercurrent extraction and tentatively identified as the mono-glucuronide. This metabolic product was readily hydrolyzed with β -glucuronidase, yielding active Chloromycetin.

No increase was observed in the urinary excretion of aryl amines in man following the administration of Chloromycetin, but lower animals such as the mouse, rat, and guinea pig excreted aryl amines in addition to inactive nitro compounds. *In vitro* studies with tissues of the rat and guinea pig demonstrated the presence of enzyme systems which converted Chloromycetin to inactive nitro compounds and also reduced the nitro groups to produce aryl amines.

Chloromycetin in Salmonellosis. S. ROSS, F. G. BURKE, E. C. RICE, and J. A. WASHINGTON. Children's Hospital, Washington, D. C.

Five cases of salmonellosis have been treated or are being treated with Chloromycetin at Children's Hospital. Two of these five cases are carriers who have failed to respond to streptomycin, aureomycin, or aerosporin during the past four months. The efficacy of Chloromycetin in these cases is too early to evaluate. The stool cultures are now negative for the first time in four months; however, insufficient time has elapsed for an adequate follow-up. The dose used was 125 mgm. every four hours orally for 10 to 14 days.

A third case had a positive blood culture for salmonella on admission. After administration of the drug, subsequent blood cultures became sterile and a positive stool culture was not obtained at any time. The child made an uneventful recovery. A fourth case currently under treatment has shown a persistence of positive stool cultures during the first four days after initiation of the drug.

The fifth case was a critically ill child with a positive blood culture. Chloromycetin was started shortly after the positive blood culture was obtained; however, the child died within 12 hours. The duration of therapy was too short to permit any evaluation of the efficacy of the drug in this case.

It would seem thus far that Chloromycetin might be more promising in salmonellosis than any antibiotic we have used; however, a larger number of cases and a longer follow-up would be mandatory before any categorical statement could be made.

Chloromycetin¹ in Therapy of Bacillary Urinary Infection. GEORGE E. CHITTENDEN, ELWOOD A. SHARP, A. J. GLAZKO, and A. S. SCHLINGMAN. Research Laboratories, Parke, Davis and Company, Detroit, Mich.

When the clinical investigation of the antibiotic effect of Chloromycetin in bacillary urinary tract infections was begun, only fragmentary data on blood levels, urine concentrations and excretion rates were available. As a result, numerous dosage patterns were studied. Some patients were given large (3.0 Gm.) single doses and were followed by frequent urinary cultures and blood levels. Others were given 2.0 Gm. single doses followed by 0.5 to 1.0 Gm. at four to eight hour intervals.

As a result of a study of approximately 50 patients, the following facts have been determined:

- (1) Chloromycetin appears to be a safe drug, especially when given orally in a wide range of dosage patterns. Thus, in mild to moderate bacillary infections, 0.5 Gm. every six to eight hours around the clock will maintain a satisfactory therapeutic blood level. In severe cases, a large initial dose (1.0 to 3.0 Gm.) followed by maintenance doses of 0.5 Gm. every six to eight hours will be adequate.
- (2) Therapy may be continued for a long period of time with no adverse pathological reactions. In one case, approximately 54.0 Gm. were given continuously without toxic or allergic reactions.
- (3) Since our studies have indicated the frequent presence of a multiplicity of organisms (Gram-positive and Gram-negative), treatment of urinary infections with sulfadiazine or penicillin in addition to Chloromycetin may be indicated.

Aureomycin and Chloramphenicol: Use in Typhus, Typhoid and Brucellosis. VERNON KNIGHT, WALSH McDERMOTT, and FRANCISCO RUIZ-SANCHEZ. Department of Medicine of the New York Hospital-Cornell University Medical College, New York, N. Y., and the Institute for the Experimental Study of Infections of the University of Guadalajara, Mexico.

Twenty-eight patients with typhus fever, presumably of murine variety, have been treated with aureomycin. The majority of a group of 23, treated by oral administration, received 100 to 200 mg. per Kg. per day for periods of one to two days with dramatic improvement and prompt clinical remission.

Other patients were treated with lower oral doses and by the intravenous route. These studies indicated that 50 mg. per Kg. per day orally for 36 to 48 hours was the lowest limit of effective dosage and that a dose of 25 mg.

¹ Trade-Mark Parke, Davis and Company for chloramphenicol.

per Kg. per day for 36 hours was sub-effective. Sub-effective and effective doses of 400 mg. and 1,000 mg. intravenously respectively in a 48 hour period of time were observed.

It was concluded that aureomycin constitutes highly effective therapy for typhus fever and that an approximate oral-intravenous ratio of effectiveness of one to five exists in this disease.

Twenty-three patients clinically ill with brucellosis, of whom nine had positive blood cultures for *B. melitensis*, were treated with aureomycin and chloramphenicol. The treated groups were almost equally divided and received similar doses of these drugs for periods averaging about eight days in doses ranging from 50 to 100 mg. per Kg. per day. Prompt clinical remission occurred in both groups after therapy. No significant differences were observed in the degree and duration of fever after therapy, although the clinical impression was gained that slightly more chloramphenicol than aureomycin was necessary to produce comparable results in the treatment of this infection. Frequent relapses make necessary further study of dosage regimens and evaluation of therapy in terms of permanent remission.

Forty-six patients with typhoid fever, of whom 35 were bacteremic, were treated with chloramphenicol or aureomycin. Dosages of aureomycin ranged from 50 to 500 mg. per Kg. per day for approximate eight day intervals. Chloramphenicol was given in most cases in doses of 100 mg. per Kg. per day for a similar period of time.

Clinical observations revealed that chloramphenicol was far superior to aureomycin in the treatment of typhoid fever, and this was in conformity with the previously reported good results with chloramphenicol. A definite, but slight effect was observable, however, in the aureomycin-treated patients.

Polypeptide Antibiotics

*Chemical Studies of Circulin.*¹ D. H. PETERSON and L. M. REINEKE. Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Circulin, an antibiotic more active against Gram-negative than Gram-positive organisms, was isolated and purified from the fermentation liquors of a non-hemolytic strain of *B. circulans* by Peterson and Colingsworth, working in cooperation with Murray, Tetrault, Kaufmann and Koffler.

Circulin, like the polymyxins, is a basic polypeptide, but differs from them in amino acid composition and is inactivated by lipase. The active principle was considered pure when repeated carbon chromatography and regeneration from its salts failed to raise the activity or alter its chemical composition. Circulin readily forms salts such as the hydrochloride, picrate, reineckate and helianthate. The antibiotic is insoluble in the water immiscible sol-

vents but is very soluble in water and less soluble in the lower alcohols. Circulin gives a negative ninhydrin-carbon dioxide reaction. Nevertheless, it contains free amino groups as exhibited by the Van Slyke and formalin reactions as well as by its reaction with 2,4-dinitrofluorobenzene.

An optically active isomer of pelargonic acid was isolated from the acid hydrolysate of circulin. Paper strip chromatography, microbiological and isolation studies of this hydrolysate demonstrated the presence of L- α , γ -diaminobutyric acid, L-threonine and D-leucine. From 300 mg. of acid hydrolysate it was possible to separate the amino acids by powdered cellulose partition chromatography in sufficient quantities to identify the constituents by various chemical means.

Paper strip chromatography reveals that the only free basic amino groups of circulin are due to the γ -amino group of α , γ -diaminobutyric acid.

Studies on Circulin. P. A. TETRAULT, H. KOFFLER, O. W. KAUFMANN, and L. Y. QUINN. Laboratories of Bacteriology, Department of Biological Sciences, Purdue University, Lafayette, Ind.

The discovery of an antibiotic which is very active against Gram-negative bacteria and is related to the polymyxins was recently announced by Murray and Tetrault (*Proc. Soc. Am. Bact.*, 1948, 1, 20). This antibiotic is produced by *Bacillus circulans* Q-19 and was therefore named *circulin* in a paper in press which also summarizes information on its assay, isolation, and general properties (Murray, Kaufmann, Tetrault, Koffler, Peterson, and Colingsworth, *J. Bact.*, 57, in press). This antibiotic is not identical with the highly toxic product of *Bacillus krzemieniewski* M-14 which is being studied at the Venereal Disease Research Laboratory and which until recently was also named circulin (McLeod, *J. Bact.*, 1948, 56, 749-754).

Yields which are approximately equivalent to from 50 to 100 mg. of pure circulin sulfate per 100 ml. of medium can be obtained during the submerged growth of *Bacillus circulans* Q-19 in a medium which contains the following: 2 per cent yeast extract, 2 per cent dextrin, 1 per cent $(\text{NH}_4)_2\text{SO}_4$, 0.4 per cent KCl, 0.02 per cent KH_2PO_4 , and 1.6 per cent CaCO_3 . The following chemical changes occurred during a representative fermentation: Bacterial growth proceeded fairly rapidly after inoculation, especially so after 12 hours. Maximum cellular development was reached approximately after 36 hours. Highest circulin yields were obtained 12 hours later. At that time the cells had begun to autolyze. Organic nitrogen compounds appeared to be used in preference to ammonia nitrogen. After 36 hours almost all of the organic nitrogen had been utilized while the concentration of ammonia nitrogen remained relatively unchanged. The utilization of dextrin paralleled bacterial growth. After 36 hours, when a maximum population level had been reached, dextrin was no longer used, although approximately one-fourth of the original amount still remained. While utilizing dextrin the culture produced lactic acid which is then kept on using until it reaches maximum growth.

¹ In a recent note to the *J. Bact.* (In Press) the name Circulin was withdrawn in favor of Mucoidin for an antibiotic described by Charlotte McLeod, *J. Bact.*, 1948, 55, 749.

The pH values reflected these chemical changes. A drop in pH from 7.8 to 6.4 accompanied the production of lactic acid; when the lactic acid was utilized and nitrogen compounds were released, the pH rose from 6.4 to 7.4, and then more gently to 7.8, a pH value reached after 73 hours. Preliminary experiments indicated that circulin protects white mice against infections caused by *Salmonella typhosa*, *Klebsiella pneumoniae*, and *Vibrio cholerae*. The amounts of circulin necessary to accomplish this were from one-seventh to one-fourth as large as the amounts lethal to half of the mice tested.

A Consideration of the Toxicity of Bacitracin Following Systemic Administration. FRANK LAMONT MELENEY and BALBINA JOHNSON. Columbia University, College of Physicians and Surgeons, New York, N. Y.

There is no evidence of toxicity or allergy from the local injection of bacitracin in the treatment of localized surgical infections from the surface application of bacitracin, or from mouth administration of single doses as high as 250,000 units. Nevertheless, evidences of kidney irritation have appeared following the intramuscular injection of doses ranging from 10,000 to 100,000 units administered every four to six hours. These toxic effects have varied strikingly with the different manufactured lots and with different manufacturing methods. This was not fully recognized until June, 1948.

Since July, precautions have been taken to use only those lots which have demonstrated an LD 50 of 300 or more for a 20-gram mouse. In the last eight months, 60 or more cases have been treated successfully with these lots without any evidence of serious kidney damage. This report covers more than 200 cases which clearly reveal not only the variations in toxicity but the safety and effectiveness of lots which have demonstrated, by the FDA tests, an LD 50 of 500 for 20-gram mice. Sixty per cent of these cases have been treated with bacitracin because they failed to respond to other methods of antibacterial therapy and more than half of these have then responded to bacitracin.

Recent experience indicates that there is an increasing number of bacterial infections which do not respond to penicillin and the causative organisms are frequently found to be resistant to penicillin but susceptible to bacitracin. These facts clearly indicate the need for an antibiotic with the attributes of bacitracin.

Bacitracin Therapy of Experimental Staphylococcal Meningitis in the Dog. PAUL TENG and FRANK LAMONT MELENEY. Columbia University, College of Physicians and Surgeons, New York, N. Y.

Early experiments in normal animals demonstrated that bacitracin was held back by the blood-brain barrier and reached the cerebrospinal fluid in only about 10 per cent of the concentration attained in the blood, following intravenous or intramuscular injection. However, this concentration was considerably increased in the presence of local inflammation.

Following the intracisternal injection of 3 cc. of saline containing 10,000 units of bacitracin in normal dogs,

there was some initial stiffness of the neck and pleocytosis; but the animals appeared normal as soon as they recovered from the anesthetic and there was no evidence of injury to the brain or cord.

In a series of 16 dogs in which meningitis was produced by the intracisternal injection of 500 million staphylococci, one-half of the animals were retained as controls while the other half were treated with the intracisternal injection of bacitracin at varying intervals following the inoculation of the organisms. In all of the animals, symptoms of meningitis rapidly developed and in the controls it was invariably fatal in from four and one-half to 37 hours, but the treated animals survived if treatment was begun within three hours following the inoculation of organisms. The intracisternal treatments varied from one to three in number, ranging from 4,000 to 10,000 units contained in 3 cc. of saline. Intracisternal treatment was supplemented by intramuscular injections of bacitracin varying from one to eight in number, ranging from 10,000 to 50,000 units. Intramuscular injections alone, containing 25,000 units every six hours, in four animals failed to save any of them.

These experiments indicate the trial of bacitracin by the intrathecal route in patients with meningitis due to organisms susceptible to bacitracin.

Parenteral Bacitracin in Surgical Infections. ALFRED B. LONGACRE, ROBERT M. WATERS, and FLORENCE EVANS. Department of Surgery, Louisiana State University, School of Medicine, New Orleans, La.

Bacitracin is an antibiotic derived from the Tracey strain of *Bacillus subtilis*. It exerts its greatest antibiotic action against species and strains of Gram-positive cocci. However, it is also an effective antibiotic against the clostridia organisms and also many strains of spirochetes including *T. pallidum*. As with the other antibiotics its full range of antibiotic activity will only be determined by further investigation of its inhibiting action on various strains of bacteria.

In this study of 50 cases of infection, bacitracin was an effective therapeutic agent in 33 or 65 per cent of the cases. It was administered parenterally in varying doses, ranging from 2,000 to 52,000 units every six hours. Some of the cases also received bacitracin locally in addition to the parenteral dose. The types of infection studied include those of cellulitis, deep tissue abscesses, infected wounds, furuncles and carbuncles, human bite and other miscellaneous infections. All bacteria recovered from the infections were tested for bacitracin sensitivity. In 25 instances the flora contained at least one bacitracin-sensitive organism and in this group 19 or 76 per cent responded favorably, whereas in the group of eight cases with only resistant strains, only three or 37.5 per cent had a favorable response. There were 16 cases, mostly those of cellulitis, from which no cultures were obtainable. In this last group 12 or 75 per cent had an excellent or good response.

The usefulness of parenteral bacitracin is at present restricted because of its toxic reactions. In this series, 16 cases demonstrated changes indicative of a possible lower

nephron syndrome. However, in only two instances were these changes of sufficient magnitude to be considered serious, and in no instance did a reaction persist after cessation of administration of the antibiotic. The exact factor responsible for these reactions is not known but the accumulated evidence suggests that it probably is not in the bacitracin itself but is a part of the mixture. The incidence of reactions varied with the different lots of bacitracin and did not appear to vary with the size of the dose or type of infection.

Even though 50 cases is a very small number of cases, the clinical response in those responding favorably indicates that bacitracin is an antibiotic of definite therapeutic value in cases which are caused by bacteria sensitive to it.

The Use of Aerosporin in Specific and Non-specific Enteritis in Infants and Children. FREDERIC G. BURKE, SIDNEY ROSS, E. CLARENCE RICE, and JOHN A. WASHINGTON. Children's Hospital, Washington, D. C.

Forty cases of enteritis were treated with aerosporin. These included 18 cases of non-specific enteritis, 16 cases

of shigella, 40 cases of salmonella enteritis and two cases of typhoid fever. The dose employed was 2 to 3 mgms. per kg. of body weight every four hours by mouth for seven to 15 days. The aerosporin sensitivity ranged from 0.02 to 0.08 μ g. per ml. Within 4° to 72 hours there was a marked diminution of the stool bacterial flora but no particular benefit was noted throughout the course of the non-specific diarrhea. In 14 of the 16 shigella cases a cure was noted and in two no significant effect was observed.

The value of aerosporin in salmonella was equivocal and the drug was given intramuscularly and by mouth. Toxic manifestations intervened in two of the cases and precluded adequate evaluation. There was no striking effect of the drug in the two cases of typhoid fever.

Following intramuscular injection toxic reactions included local pain, fever, malaise, and leukocytosis. Nephrotoxic reactions were noted in cases given the drug by mouth and intramuscularly and consisted of azotemia, albuminuria, white cells and casts in the urinary sediment. The high incidence of toxic manifestations of the drug would preclude its widespread usefulness.

THE SERUM PROTEINS IN INFECTIOUS MONONUCLEOSIS. ELECTROPHORETIC STUDIES

By KENNETH STERLING¹

(From the Department of Medicine, the University of Chicago)

(Received for publication February 17, 1949)

Previous investigations have shown the very frequent occurrence in infectious mononucleosis of abnormal findings on liver function tests (1-7), and of hepatic involvement on histologic examination of both autopsy (8-11) and biopsy material (1, 12-14) in cases with and without jaundice.

Alterations observed in the composition of the serum proteins (2, 4, 6, 15, 16a) appear to be compatible with the occurrence of positive cephalin-cholesterol flocculation and thymol turbidity

tests, notwithstanding the various interpretations advanced to explain the mechanisms of these tests (16-22).

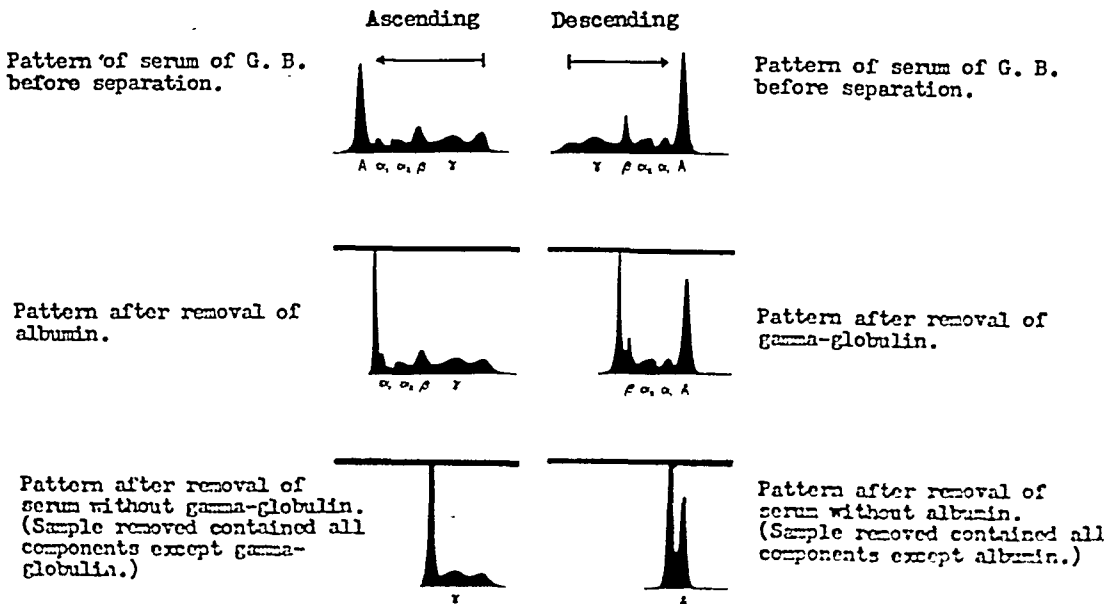
The present work was undertaken to study in detail the serum proteins in infectious mononucleosis by the electrophoretic method and to examine separated protein fractions for the presence of heterophile antibodies.

METHODS

The electrophoretic runs were performed with the standard apparatus and technique described by Longworth (23) using sodium diethylbarbiturate (veronal)

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SEPARATION OF SERUM PROTEIN FRACTIONS



The four samples obtained from each serum were: (1) separated albumin, (2) serum without albumin, (3) serum without gamma-globulin, (4) separated gamma-globulin.

FIG. 1

The albumin peaks are indicated by "A"; the alpha-1, alpha-2, beta, and gamma globulins are designated by their respective Greek letter prefixes. The unlabelled peaks at the extreme left and right are not protein components but are still very prominent in the early stages of the run. The sharp peak reaching up at the bottom is due to the sharp gradient in the potential at the level of the capillary after eight hours of separation.

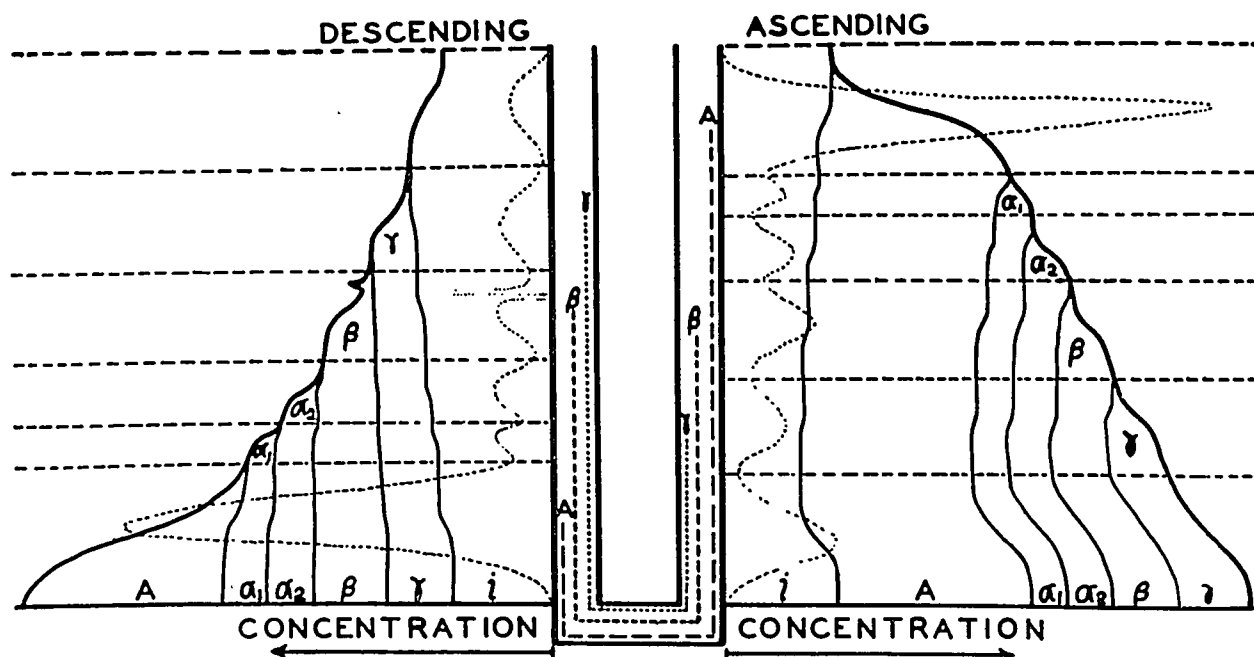


FIG. 1a. CONCENTRATION DIAGRAM OF SERUM COMPONENTS IN CELL AFTER ELECTROPHORESIS *

Buffer salt concentration is designated by "i," albumin by "A," and the alpha-1-, alpha-2-, beta-, and gamma-globulins by their respective Greek letter prefixes.

The solid line curves indicate the concentrations of components. The dotted lines are outlines of electrophoretic patterns as photographed, and show refractive index gradients caused by the components. Since the dotted lines represent the slopes (first derivatives) of the solid line curves, the areas under the peaks of the electrophoretic patterns give the relative concentrations of protein constituents.

buffer of pH 8.6 and ionic strength 0.1. After dialysis against the buffer the serum was diluted with buffer solution to four times its original volume prior to dialysis.

Electrophoresis was carried out at approximately 1.5°C. for 120 minutes at a potential gradient of 7.7 volts per centimeter. Photographs were taken by the schlieren scanning method.

In the estimation of the fractions as per cent of the serum proteins both ascending and descending patterns were used, the areas being divided by ordinates from the nadirs between peaks to the base line. The quantities of the fractions as grams per cent represent the products (24) of the percentages and the total proteins from the conventional Kjeldahl method, neglecting recent theoretically valid refinements (25, 26).

The separation of fractions was carried out by means of a capillary pipette lowered into the electrophoresis cell by rack and pinion. In this manner four fractions were obtained. From the ascending limb the *separated albumin* layer above the other components was aspirated. The pipette tip was then lowered further into the cell to a point just above the start of the ascending gamma-globulin peak and a sample was drawn off containing all components except gamma-globulin (*serum without gamma-globulin*). From the upper part of the descend-

ing limb *separated gamma-globulin* was obtained. Lowering the pipette tip to a point just above the start of the descending albumin peak yielded a sample with all components except albumin (*serum without albumin*). All these collections were performed with careful visualization of the peaks and pipette tip by the cylindrical lens method and schlieren band method when needed. After the removal of each sample a photograph was taken to show the remaining undisturbed pattern as a check on the level of the pipette tip during collection and as an indication of the accuracy of the separation. (See Figures 1 and 1a.)

In a few instances the attempt was made to verify further the accuracy of separation by reanalyzing fractions electrophoretically in the micro-cell. (See Figure 2.)

Heterophile antibody titration (27) was done on the four fractions obtained from each serum, and also on the dialyzed buffer diluted serum (not subjected to electrophoresis) which served as a control. The titres are reported as "final serum dilutions" considering the samples of fractions or controls as representing the "undiluted serum"; actually the controls were 1:4 dilutions, and the fractions were still further diluted to an indeterminate extent because of unavoidable dilution with buffer in the separation of fractions.

Absorption of heterophile antibodies was carried out by repeated incubation of inactivated serum with packed sheep red cells.

* Diagram from Moore, D. H., and Abramson, H. A.; *Electrophoresis*, to appear in *Medical Physics* (ed., O. G. Peterson), 1950, Year Book Publishers, Inc., Chicago, Ill.

CASE MATERIAL

As normal controls the fasting sera of ten volunteer medical students and physicians were examined.² All were normal with respect to total proteins, albumin and globulin, alkaline phosphatase, bromsulfalein retention, hippuric acid excretion, prothrombin time, cephalin-cholesterol flocculation and thymol turbidity tests.

Seven hospitalized cases of infectious mononucleosis where the diagnosis was considered certain were selected for study. All exhibited definite enlargement of lymph nodes as well as other suggestive clinical manifestations. At some time during the hospital course each patient showed lymphocytosis as high as 50% or greater, including cells considered characteristic of the disease. Heterophile antibody titres of 1:512 or higher were present in all cases.

Two patients (T. S. and L. M.) had moderate jaundice, and one (R. S.) had slight jaundice, subsiding at the time his serum was studied. The serum bilirubin was normal in the other four cases.

To compare the findings with those in infections of approximately similar severity but without known hepatic disorder, two hospitalized cases of upper respiratory tract infection were chosen at random: one beta-hemolytic

² These sera form part of a larger study of liver disease being done in collaboration with Drs. William E. Ricketts, Joseph B. Kirsner, and Walter L. Palmer.

MICRO-CELL ELECTROPHORESIS

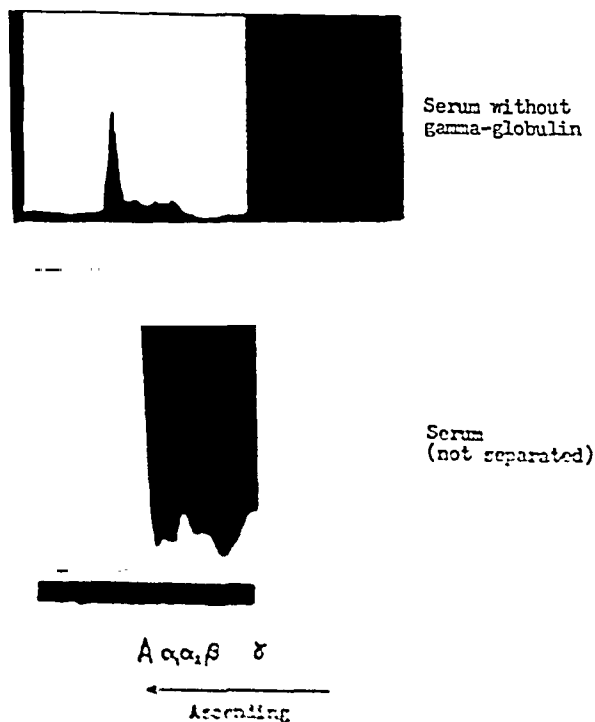


FIG. 2

streptococcal pharyngitis and one "acute upper respiratory tract infection, type unspecified," both with negative heterophile antibody determinations and thymol turbidity tests.

For comparison with known hepatic disease the following three cases² were studied:

- C. G., subsiding infectious hepatitis followed with serial punch biopsies of the liver;
- E. H., fatal hepatitis of probable virus etiology with progressive downhill course characterized by jaundice, edema, ascites, and hemorrhagic phenomena, and showing at autopsy massive hepatic necrosis with regeneration of pseudolobules;
- M. S., Laennec's cirrhosis with jaundice, ascites, and edema, the diagnosis being confirmed by liver punch biopsy.

RESULTS

Normal Sera

The electrophoretic and chemical data on the serum proteins of the ten normal volunteers with negative liver function tests are listed in Table I. The findings are comparable with those of other workers.

Infectious Mononucleosis Sera

The electrophoretic and chemical data on the serum proteins of the infectious mononucleosis patients with results of simultaneous liver function tests are listed in Table II.

In both the per cent compositions and the absolute amounts there were diminutions of the albumin fractions and elevations of the gamma-globulin fractions as compared with the normal sera. These deviations appear to be significant in all but the non-jaundiced patient D. C. who was also the only case with negative cephalin-cholesterol flocculation and thymol turbidity tests, and in whom the only normal alkaline phosphatase occurred among the six determinations done. The studies on this patient were done on the 24th day of illness when he had become afebrile three days before discharge.

Less pronounced and less frequently observed alterations were the elevations of the alpha-1-globulin and beta-globulin fractions.

The jaundiced cases, T. S. and L. M., exhibited somewhat more pronounced changes in serum protein composition than most of the non-jaundiced cases, but the non-jaundiced patient G. H. showed the most abnormal patterns of all, as well as the highest thymol turbidity. It will be noted from Table II that the two patients who were studied a second time, ten days later, had less

TABLE I
NORMAL SERA

	Electrophoretic data										Howe sodium sulfate				
	per cent					grams per cent					grams per cent				
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ	A/G	A/G	Alb	Glob	Tot
1.	63.3	2.5	8.8	12.0	13.5	4.52	0.18	0.63	0.86	0.96	1.73	2.43	5.06	2.08	7.14
2.	61.1	3.3	9.1	12.7	13.9	4.70	0.25	0.71	0.98	1.07	1.57	2.38	5.43	2.28	7.71
3.	60.0	4.7	10.6	14.3	10.4	3.93	0.31	0.70	0.94	0.68	1.50	2.32	4.58	1.98	6.56
4.	60.6	3.6	8.5	13.2	14.1	4.07	0.24	0.57	0.89	0.95	1.54	2.95	5.02	1.70	6.72
5.	61.3	4.6	9.5	13.4	11.2	4.18	0.31	0.65	0.91	0.77	1.58	2.67	4.96	1.86	6.82
6.	55.0	4.6	12.9	13.2	14.3	3.80	0.32	0.89	0.91	0.99	1.22	1.93	4.55	2.36	6.91
7.	56.8	5.3	11.3	13.7	12.9	4.02	0.38	0.80	0.97	0.91	1.32	1.77	4.52	2.56	7.08
8.	64.8	4.5	9.2	9.9	11.6	4.53	0.32	0.64	0.69	0.81	1.84	2.41	4.94	2.05	6.99
9.	58.6	3.6	9.6	12.1	16.1	4.06	0.25	0.66	0.84	1.11	1.42	1.97	4.59	2.33	6.92
10.	61.9	3.0	7.3	13.2	14.6	4.48	0.22	0.53	0.95	1.06	1.63	2.55	5.20	2.04	7.24
<u>AVERAGE</u>	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93	1.52	2.31	4.89	2.12	7.01
Standard Deviation	2.8	0.8	1.5	1.2	1.7	0.29	0.06	0.10	0.08	0.13	0.18	0.34	0.30	0.24	0.30

jaundice and other clinical symptoms were subsiding, but the abnormalities of the serum proteins and liver function tests were still present.

Upper Respiratory Tract Infections

There were no significant deviations from normal in the per cent compositions of the serum proteins in the two hospitalized cases of upper respiratory tract infection.

The albumin depression and gamma-globulin elevation were least pronounced in C. G., the case of subsiding infectious hepatitis, where the abnormalities were somewhat less marked than in most of the infectious mononucleosis cases. These deviations from normal were more pronounced in E. H., the case of fatal hepatitis, and were most striking in M. S., the case of Laennec's cirrhosis, where the gamma-globulin actually exceeded the albumin.

	Albumin	α_1 -glob.	α_2 -glob.	β -glob.	γ -glob.	A/G ratio
Beta-hemolytic streptococcal pharyngitis	58.0%	5.2%	10.6%	11.6%	14.6%	1.38
Upper resp. tr. infect., type unspecified	62.9%	4.3%	8.7%	11.5%	12.6%	1.70

Known Hepatic Disease

The findings in the three cases of histologically proven liver disease are shown in Figure 3, together with data from normal and infectious mononucleosis sera.

Heterophile Antibody Titres of Separated Fractions

After electrophoresis each infectious mononucleosis serum was separated into the following four fractions:

1. Separated albumin
2. Serum without albumin
3. Serum without gamma-globulin
4. Separated gamma-globulin.

The dialyzed buffer diluted serum (not subjected to electrophoresis) served as a control.

The results of the heterophile antibody titrations are listed in Table III. The figures express "final serum dilutions," considering the samples of fractions or controls as representing the "undiluted serum"; actually the controls were 1:4 dilutions and the fractions were still further diluted to an indeterminate extent.

As shown in Table III, heterophile antibodies were demonstrated in the *separated gamma-globulin* in every case, and were never present in the *separated albumin*. Heterophile antibodies were absent from the *serum without gamma-globulin* in seven of the ten experiments recorded, but present in three.

The low titres in the *separated gamma-globulin* samples in comparison with the controls were presumed due to the unavoidable dilution in the process of fractionation. To test the supposition that these samples contained minute amounts of protein, micro-Kjeldahl determinations were done on three samples of *separated gamma-globulin* with the following findings (after subtraction of buffer nitrogen):

T. S., 12th day—30.8 mg. nitrogen per 100 cc.
D. C., 24th day—22.4 mg. nitrogen per 100 cc.
M. W., 11th day—18.2 mg. nitrogen per 100 cc.










Assuming a nitrogen factor of 6.25, these samples contained approximately one-tenth to one-seventh the gamma-globulin concentration of their respective undiluted whole sera.

Attention was directed toward the occurrence of heterophile antibodies in a few of the samples of *serum without gamma-globulin*. It was suspected that the separations might not be accurate, but

TABLE II

Day of illness indicated below initials of each patient. Values for each serum protein fraction expressed as grams per cent, and per cent of total proteins. Alkaline phosphatase activities in Bodansky units.

INFECTIOUS MONONUCLEOSIS SERA

Ascending Descending		Electrophoretic data						Howe sodium sulfate				bilirub		ceph	thym	alk. phos.
		Alb	α_1	α_2	β	γ	A/G	A/G	Alb	Glob	Tot	dir	tot			
	T.S. 12th day	3.44 46.8%	0.49 6.7%	0.75 10.2%	0.91 12.3%	1.76 23.9%	0.88	1.32	4.18	3.17	7.35	1.3	2.4	4+	10.7	12.8
	T.S. 22nd day	3.26 45.7%	0.44 6.1%	0.51 7.2%	1.23 17.2%	1.70 23.8%	0.84	1.44	4.21	2.93	7.14	0.5	0.9	3+	11.3	8.2
	L.M. 13th day	3.24 50.6%	0.25 3.9%	0.58 9.0%	1.16 18.1%	1.19 18.5%	1.02	1.66	4.01	2.41	6.42	1.8	2.9	4+	11.8	21.8
	L.M. 23rd day	3.84 56.7%	0.34 5.0%	0.45 6.6%	1.06 15.7%	1.09 16.0%	1.31	1.92	4.46	2.32	6.78	0.7	1.3	3+	9.8	13.5
	R.S. 17th day	3.51 50.7%	0.18 2.6%	0.83 12.0%	1.02 14.7%	1.39 20.0%	1.03	1.42	4.07	2.66	6.93	0.9	1.3	4+	13.0	9.4
	D.C. 24th day	3.90 54.4%	0.52 7.2%	0.88 12.2%	0.86 12.0%	1.02 14.3%	1.19	2.03	4.81	2.37	7.18	0.3	0.5	2+	3.9	4.2
	D.D. 17th day	3.88 53.1%	0.34 4.6%	0.81 11.1%	1.15 15.8%	1.12 15.4%	1.13	2.17	5.00	2.30	7.30	0.1	0.5	4+	9.1	
	G.B. 16th day	2.91 42.2%	0.52 7.6%	0.73 10.5%	1.22 17.7%	1.52 22.0%	0.73	1.30	3.90	3.00	6.90	0.2	0.3	4+	13.4	
	M.W. 11th day	3.32 48.9%	0.51 7.5%	0.85 12.5%	0.98 14.4%	1.14 16.7%	0.96	1.62	4.20	2.60	6.80	0.2	0.5	3+	8.2	
	AVERAGE	3.49 48.9%	0.40 5.7%	0.71 10.1%	1.07 15.3%	1.33 18.6%	1.00	1.62	4.31	2.66	6.96					

this appeared quite unlikely on study of the photographs taken after the steps of the separation procedure in the manner of Figure 1.

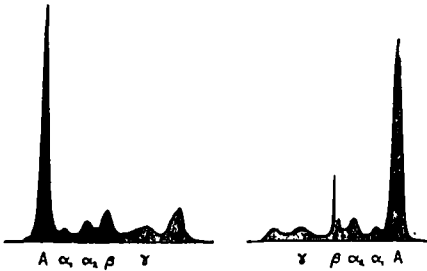
Although L. M. on the 13th day of illness

showed heterophile antibodies in the *serum without gamma-globulin*, there were none in the analogous sample on the 23rd day, as shown in Table III. Lacking more serum from the 13th day

NORMAL SERUM

Patterns of normal serum #5; figures are averages of ten normal sera.

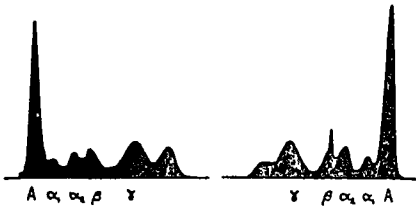
	Alb	α_1	α_2	β	γ	A/G
per cent	60.3	4.0	9.7	12.8	13.2	1.52
grams per cent	4.23	0.28	0.68	0.89	0.93	
Howe sod. sulfate	4.89	Glob-2.12		Tot-7.01		2.31



INFECTIOUS MONONUCLEOSIS

Patterns of T.S., 12th day of illness; figures are averages of data from the cases studied.

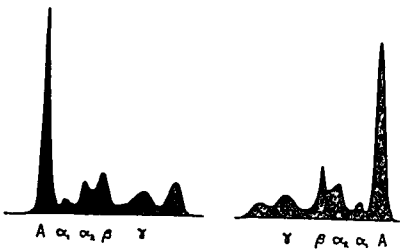
	Alb	α_1	α_2	β	γ	A/G
per cent	49.9	5.7	10.1	15.3	19.0	1.00
grams per cent	3.48	0.40	0.71	1.07	1.33	
Howe sod. sulfate	4.31	Glob-2.66		Tot-6.98		1.62



INFECTIOUS HEPATITIS, MILD

Patterns and figures of C.G. with subsiding hepatitis, verified by serial punch biopsies of the liver.

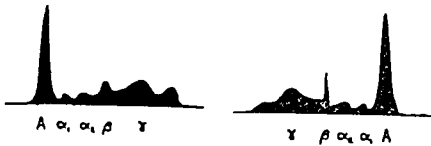
	Alb	α_1	α_2	β	γ	A/G
per cent	50.2	4.6	12.9	16.5	15.8	1.01
grams per cent	3.54	0.32	0.91	1.16	1.11	
Howe sod. sulfate	4.50	Glob-2.54		Tot-7.04		1.77



FATAL HEPATITIS

Patterns and figures of E.H. with rapid course of fatal hepatitis, confirmed at autopsy. Virus etiology probable.

	Alb	α_1	α_2	β	γ	A/G
per cent	43.5	4.4	6.8	14.0	31.3	0.77
grams per cent	2.08	0.21	0.32	0.67	1.49	
Howe sod. sulfate	2.33	Glob-2.44		Tot-4.77		0.96



LAENNEC'S CIRRHOSIS

Patterns and figures of M.S. with decompensated cirrhosis; jaundice, ascites, and edema; liver punch biopsy done.

	Alb	α_1	α_2	β	γ	A/G
per cent	31.0	5.0	8.0	17.0	39.0	0.45
grams per cent	2.01	0.32	0.52	1.10	2.53	
Howe sod. sulfate	2.56	Glob-3.92		Tot-6.48		0.65

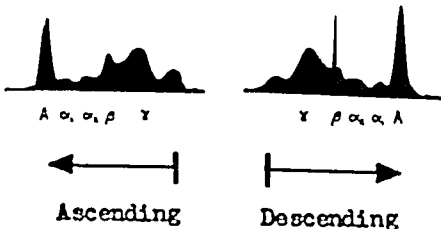


FIG. 3

The albumin peaks are indicated by "A"; the alpha-1-, alpha-2-, beta-, and gamma-globulins are designated by their respective Greek letter prefixes. The unlabelled peaks are not protein components but are stationary or semi-stationary due to gradients of buffer salt.

TABLE III

As explained in the text, the titres below cannot be regarded as precise quantitative expressions because of unavoidable (and variable) dilution in the process of fractionation.

HETEROPHILE ANTIBODY TITRES OF SEPARATED FRACTIONS

	Buffer diluted serum (CONTROL)	Separated albumin	Serum without albumin	Serum without gamma globulin	Separated gamma globulin
T.S. 12th day	1:256	neg.	1:256	neg.	1:128
T.S. 22nd day	1:256	neg.	1:64	neg.	1:16
L.M. 13th day	1:512	neg.	—	1:16	1:32
L.M. 12th day	1:512	neg.	1:128	1:8	1:32
L.M. 23rd day	1:256	neg.	1:128	neg.	1:16
R.S. 17th day	1:256	neg.	1:128	neg.	1:16
D.C. 24th day	1:64	neg.	1:16	neg.	1:8
D.D. 17th day	1:512	neg.	1:256	neg.	1:8
G.B. 16th day	1:128	neg.	1:64	neg.	1:16
M.W. 11th day	1:128	neg.	1:32	1:8	1:8

to repeat the original experiment, serum from the 12th day was used instead, with similar findings. This duplication of results also argues against technical error in the separation.

As a further check, micro-cell electrophoretic runs (see Figure 2) were performed on the three samples in question. Because part of each sample (already dilute) had been used for heterophile antibody titration, it was necessary to add buffer to obtain the 2 cc. required for a micro-cell run. Consequently, the peaks were quite low and the patterns could be interpreted as no more than suggestive evidence of the absence of gamma-globulin.

Absorption of Heterophile Antibodies

By absorption with sheep red cells the heterophile antibody titre of the serum of G. B. (19th day of illness) was reduced from 1:2048 to 1:32.

The electrophoretic patterns before and after antibody absorption were practically identical.

DISCUSSION

The serum protein compositions of the infectious mononucleosis patients showed definite diminutions of the albumin fractions and elevations of the gamma-globulin fractions as compared with normal sera. Less pronounced and less frequently observed deviations from normal were the elevations of the alpha-1-globulin and beta-globulin fractions.

The cases studied were limited to hospitalized patients in whom the diagnosis could be made with certainty; it cannot be asserted that an unselected series would necessarily have shown as pronounced changes.

Numerous investigators have observed generally

	Albumin	Alpha-1	Alpha-2	Beta	Gamma
Before absorption (titre 1:2048)	43.4%	8.0%	17.1%	10.7%	20.8%
After absorption (titre 1:32)	43.2%	7.7%	18.1%	10.5%	20.5%

similar if more conspicuous abnormalities in hepatitis and cirrhosis (16, 18, 28-33). As suggested by Figure 3 these changes in infectious mononucleosis may bear some relationship to the type if not the degree of hepatic dysfunction.

The cephalin-cholesterol flocculation and thymol turbidity tests may merely reflect directly the abnormalities of the serum proteins (16-22). The concomitance, however, of occasional hyperbilirubinemia, frequent elevation of alkaline phosphatase activity (2, 5, 6), and histopathologic evidence of liver involvement (1, 8-14) makes plausible the association of disturbed hepatic function and the alterations of the serum proteins. Such a conception is not in any sense incompatible with the probable extra-hepatic *origin* of serum globulins.

That the widespread pathologic involvement of the lymph nodes and the foci of mononuclear cell infiltration in various organs may be directly responsible for changes in the serum proteins is an attractive hypothesis. This idea has been advocated on the basis of electrophoretic findings in infectious mononucleosis and the blood dyscrasias, the latter group exhibiting rather varied alterations (15).

The possibility that the changes in the serum proteins in infectious mononucleosis may be related to the infectious process *per se* deserves consideration in the light of somewhat similar abnormalities described in infectious diseases such as syphilis, tuberculosis, typhus, malaria, lymphogranuloma venereum, leprosy, kala-azar, and others (34). It must, however, be noted that most of the above conditions are characterized by chronic proliferative granulomata or by widespread cellular destruction, hence are not too closely analogous to infectious mononucleosis. On the other hand, in lobar pneumonia there are conspicuous elevations of the alpha-2-globulins usually without significant changes in the gamma-globulins (34, 35).

In an effort to compare the findings in infectious mononucleosis with those in infections of approximately similar severity but without known hepatic disorder, the sera of two hospitalized cases of upper respiratory tract infection were analyzed electrophoretically with the finding of normal patterns. It would appear that the extent to which the serum protein changes are attributable to

the non-specific effects of the infectious process is debatable.

That the gamma-globulin increase could be due to the heterophile antibodies is excluded by the experiment showing no change in electrophoretic pattern after antibody absorption. In other human diseases absorption of clinically important specific antibodies has produced no change in electrophoretic pattern, the antibodies being present in too small quantities to cause visible alterations in schlieren patterns even though easily detectable by serological methods (34, 36). Immuno-chemical studies corroborate the minute concentrations of antibodies in human serum (37).

The data on the distribution of heterophile antibodies in relation to the serum protein fractions are interpreted as indicating that they are predominantly in the gamma-globulin fraction, not always being exclusively confined to this fraction.

The occurrence of various antibodies in the gamma-globulin fractions of animals has been demonstrated repeatedly (37, 38) since the original work of Tiselius and Kabat (39) showing pneumococcal antibodies in the gamma-globulins of rabbit and monkey sera. It must, however, be recalled that Tiselius and Kabat found in the horse that pneumococcal antibodies migrated between the beta- and gamma-globulins forming a separate peak. Since then equine antibodies to diphtheria toxin, tetanus toxin, and others have been found outside the gamma-globulin fraction (37, 38). Virus-neutralizing antibodies to Venezuelan and Western equine encephalomyelitis have been demonstrated in both the beta- and gamma-globulins of rabbit sera (40).

The extensive work of Enders (41) on the products of human plasma fractionation revealed that Fraction II, composed of almost pure gamma-globulin, contained in high concentration antibodies reacting with diphtheria toxin, streptococcal erythrogenic toxin, influenza A virus, mumps virus, and the H-antigen of *E. typhosa*. On the other hand, isoagglutinins and antibody reacting with the O-antigen of *E. typhosa* were recovered from Fraction III-1, containing mostly beta-globulin and less gamma-globulin. Furthermore, smaller amounts of the antibodies concentrated in Fraction II were found in other fractions free of gamma-globulin. Subsequently, division of human gamma-globulin into sub-fractions with differ-

ing antibody contents has been reported (42).

Electrophoretic separation of sera from allergic patients has suggested that ragweed and rabbit antibodies (or reagins) are confined to the gamma-globulin fraction (43).

In a study employing electrophoretic, ultracentrifugal, and immuno-chemical methods the Wassermann antibody (or reagin) has been clearly shown to have a mobility intermediate between beta- and gamma-globulins and overlapping both (44).

It is hoped that the present findings on heterophile antibodies in infectious mononucleosis will be supplemented by further physico-chemical studies.

SUMMARY

1. Electrophoretic analyses of the serum proteins of seven cases of infectious mononucleosis revealed deviations from the normal composition in both jaundiced and non-jaundiced patients.

2. The principal abnormalities observed were diminutions of the albumin fractions and elevations of the gamma-globulin fractions.

3. Less pronounced and less frequently observed alterations were elevations of the alpha-1-globulin and beta-globulin fractions.

4. Liver function tests showed abnormalities in six of the seven cases.

5. The alterations of the serum proteins were considered as possibly related to hepatic dysfunction, among other hypotheses entertained.

6. Separation experiments indicated the heterophile antibodies were contained predominantly in the gamma-globulin fractions of the sera, not always being exclusively confined to this fraction.

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EVIDENCE THAT RENAL SODIUM EXCRETION BY NORMAL HUMAN SUBJECTS IS REGULATED BY ADRENAL CORTICAL ACTIVITY¹

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When a normal human being is placed on a very low sodium intake, the urinary sodium decreases to very small amounts. Contrariwise, with large sodium intakes, the urinary sodium increases proportionately. In both cases, the normal body content of sodium is quickly reestablished. How is this conservation of body sodium effected?

The depletion of body sodium that occurs in adrenalectomized animals and in patients suffering from Addison's disease, and the retention of sodium with reestablishment of balance that accompanies the administration of adrenal cortical extracts, leaves no doubt about the fundamental position of a cortical hormone in the regulation of body sodium in the abnormal states just mentioned (1-5).

Since there is a paucity of information regarding the regulation of body sodium content in normal subjects who receive large variations in intake of sodium, our studies were designed to learn the role of adrenal cortical activity in this regulation in normal human beings.

Even though it is not possible to determine directly the concentration of the specific hormone in the blood, nevertheless a number of methods that serve as indirect indices of adrenal cortical activity are available. It was surmised that by creating a need to conserve body sodium in the presence of large shifts in intake, evidence of other adrenal cortical effects, especially on protein metabolism, might be elicited. If such evidence of adrenal cortical activity can be obtained by the requirement to conserve sodium, such data would greatly strengthen the hypothesis that in the normal subject this conservation of body sodium is in fact mediated through an increased or decreased adrenal cortical activity (6).

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EXPERIMENTAL

Three normal young adults were subjects for this study: one male resident physician (W. C.), age 32; one male medical student (E. W.), age 22; and one female graduate student (M. L.), age 22. The plan of study was to place the subject on an adequate caloric and protein dietary intake with a very low sodium content (approximately 200 mgs. or 9 mEq. daily) throughout the study, and at definite periods to administer orally large amounts of sodium as sodium chloride or sodium citrate.

This was accomplished by using a constant daily diet. The water intake was uncontrolled as its sodium content was found to be insignificant by analysis. The subjects went about their usual daily activities avoiding severe exertion and sweating. Urine and stool collections were made daily and the nude body weight was recorded each morning before breakfast and after emptying the bladder. A special human balance having a sensitivity of 5 gms. was used. Each 24 hour urine was collected with toluene as a preservative and was analyzed separately on the day following the collection. Stools were promptly treated with alcohol and made acid with glacial acetic acid or sulfuric acid, dried in an oven at 50° to 60° C., weighed, blended in a Waring blender and combined into two or three day periods with suitable aliquots taken for analyses. Subjects were fed from the same lot of food throughout the period of study and a sample day's diet was dried, blended and analyzed.

When a constant body weight was achieved and the urine sodium and chloride excretions had reached a minimum on this low sodium intake, 11.0 gms. or 478 mEq. of sodium were administered orally as the chloride (28.0 gms. of sodium chloride) or citrate (46.8 gms. of sodium citrate) daily for three days. Fasting blood samples were obtained with a minimum of stress at the beginning and end of the study, and before and after each period of sodium administration. Samples for potassium and carbon dioxide content were drawn under oil and the former kept refrigerated until the serum was separated from the cells with no hemolysis occurring.

Hematocrit (packed red cell volume) was estimated by the method of Wintrobe (7). Sodium analyses were done on serum, urine, diet, and stool by means of the Butler and Tuttle (8) modification of the flame photometric method of Barber and Kitch (9). For control of very low sodium content a modification was made by

aliquots and removal of potassium as well as phosphate was utilized (9). Potassium was determined in serum, urine, diet and stool by a modification of the platinum chloride micro colorimetric method of Shohl and Bennett (10). Chloride analyses of serum were done using the method of Van Slyke and Sendroy (11). Urine, stool, and diet chloride determinations were done by a modification of the Volhard-Harvey titration (12). Urinary ammonia was determined by the aeration method of Van Slyke and Cullen (13). Total urine, diet and stool nitrogen were determined by the Kjeldahl method for macro analysis (14). Urine creatinine was measured by the alkaline picrate method of Folin (15) and in subject E. W., also by the modification of Bonsnes and Taussky (16). Agreement between the very simple method of Folin and the more refined latter method was surprisingly good. The modification of Bonsnes and Taussky was also adopted for serum creatinine determinations. Blood urea nitrogen was estimated by the method of Gentzkow (17) while for urine urea the method of Barker (18) was utilized. Urine uric acid determination was done by the total color method of Buchanan, Block, and Christman (19). Carbon dioxide content of the serum was determined by the method of Van Slyke and Neill (20). Serum protein analyses were done by the micro Kjeldahl method and the steam distillation of Goebel (21). Urine, stool and diet were analyzed for phosphate by the colorimetric method of Fiske and Subbarow (22).

The aliquots of diet and stools were dry ashed in such a manner that analyses could be made for sodium, chloride, potassium, and phosphorus on a single preparation. This was accomplished by mixing the aliquot with an excess of calcium hydroxide until definitely alkaline, drying and then ashing in the muffle furnace at 400° C. for 48-72 hours. The residue was washed with water and filtered. The filtrate containing the sodium, chloride, and potassium was made up to volume in a volumetric flask from which aliquots were taken for analysis. The washed residue was later dissolved with dilute nitric acid to yield phosphate. Values for chloride of diet and stool obtained by this method were the same as values obtained by wet ashing the sample.

Inulin clearances were done by the Harrison modification of the method of Alving, Rubin, and Miller (23) following a single intravenous injection of 10-20 gms. of inulin.

The values for dietary intake of calories, carbohydrate, fat and protein were obtained from Bowes and Church (24) and U. S. Department of Agriculture Circular Number 542. The calculated values for sodium, chloride, potassium and phosphorus content of foods were obtained from McCance and Willows (25). In addition, analyses were made on complete diets (with the exception of the latter for total nitrogen, total phosphorus, sodium, chloride, and potassium). The agreement between actual analyses and calculated values was surprisingly good. This in subject M. L.'s diet the calculated and analyzed composition was respectively: nitrogen—12.3 and 11.2

gms.; sodium—8.7 and 8.3 mEq.; chloride²—21.0 and 22.8 mEq.; and potassium—89.7 and 94.0 mEq. Similarly in subject E. W.'s diet the calculated and analyzed composition was respectively: nitrogen—14.3 and 15.0 gms.; sodium—9.1 and 9.4 mEq.; chloride—24.4 and 24.9 mEq.; and potassium—100 and 100 mEq. The dietary intake figures recorded for subjects M. L. and E. W. in Tables II and III are values obtained by our analyses for sodium, chloride, nitrogen, potassium and phosphorus as no diet changes were made and all the food was eaten. In subject W. C. who frequently rejected portions of his diet, such portions were weighed and their composition as obtained from the above-mentioned food composition tables was subtracted from the total calculated content of the diet, and each day's intake is recorded in Table I.

RESULTS

The complete data of these studies are shown in Tables I, II and III.

The electrolyte and weight changes are the subject of a separate report (6). However, it is important to the present discussion to point out that with the curtailment of sodium intake to about 9 mEq. daily there occurred a sharp initial weight loss of about 2 kilograms in each subject. This weight loss was associated with a negative sodium and chloride balance (Figure 1 illustrates these changes in subject E. W.). After approximately one week of this low sodium regimen the urinary loss of sodium and chloride was reduced to extremely minute amounts (1 to 2 mEq. of sodium in the 24 hour urine specimen) and the weight became stabilized. Under the stimulus of such severe sodium restriction the kidneys conserve sodium maximally.

On administration of the large amounts of sodium (480 mEq. daily for three days) as the sodium chloride or sodium citrate salts there was with each a significant weight gain. None of the subjects showed clinical edema, however. The urinary excretion of sodium lagged in every case so that only by the last day of the sodium administration had the output attained the daily intake level.

With these changes in weight and in urinary excretion of sodium and chloride, accompanying

² Interestingly the diet analyses, not including "Lonalac," yielded nearly equivalent values for sodium and chloride. The addition of "Lonalac" (Mead Johnson and Co., Evansville, Indiana), which contains supplementary potassium chloride, resulted in the actual higher values of chloride.

TABLE I

* Values obtained from separate analysis of single loose stool.

TABLE II
Subject M. L., ♀ age 22

Day	Node No.	Serum and blood							Urine				Stool				Remarks
		Na	Cl	K	CO ₂	BUN	Prot.	Hemat.	Vol.	Creat.	NH ₄	Total N	Na	Cl	K	N	
		mg./l.	per l.	per l.	mM/l.	mg.	gms. %	% cells	cc.	gms.	mEq.	gms.	mEq.	mEq.	mEq.	gms.	
1	71,050	118.7	100.9	4.8			7.58		810	0.631	42.9	14.01	23.3	38.5	51.6	0.90	Inulin clearance 121 cc./min.
2	71,080								770	0.620	37.7	12.19	21.7	34.9	49.2	0.90	
3	70,435								535	0.610	37.5	10.97	6.1	14.1	39.2	0.90	
4	69,730								1560	0.638	35.4	9.58	31.7	33.6	40.0	1.34	
5	69,310								440	0.643	29.9	11.00	3.3	5.7	40.0	1.34	
6	69,150								410	0.610	28.4	9.59	2.6	2.7	33.2	0.7	
7	69,200								800	0.634	31.1	11.21	1.6	1.0	42.8	0.85	
8	68,924	116.6	100.0						890	0.640	33.4	9.53	1.2	0.9	41.2	0.85	
9	68,650																
10	68,190																
11	68,145																
12	68,285																
13	68,620																
14	68,610	130.1	101.1	4.7	23.9	11.4	7.46	39.0	820	0.646	28.4	9.81	152.2	152.6	87.6	1.39	Sodium chloride 479 mEq. Sodium chloride 479 mEq.
15	70,470								2100	0.651	42.0	9.76	371.2	411.2	76	1.39	
16	71,150								2530	0.624	41.6	8.75	582.9	614.4	41.6	1.39	
17	71,270	139.0	101.4	4.6	23.4	6.6	6.84	35.0	2760	0.627	35.6	7.10	201.9	188.4	30.4	1.87	Inulin clearance 123 cc./min.
18	70,020								865	0.644	23.3	8.60	53.5	62.8	67.6	1.87	
19	69,860								1190	0.690	19.9	9.12	31.0	42.8	66.8	1.87	
20	69,480								825	0.638	23.7	8.90	36.9	32.1	54.4	1.93	Onset of catamenia
21	69,120								670	0.638	23.6	10.08	3.9	8.9	53.2	1.93	
22	68,910								950	0.644	31.4	9.75	6.4	8.4	66.4	1.93	
23	68,680								1000	0.644	31.5	10.06	5.7	7.8	60.4	1.01	Sodium citrate 478 mEq. of sodium Sodium citrate 478 mEq. of sodium
24	68,670								1020	0.632	30.4	9.57	4.5	9.2	66.4	1.01	
25	68,475	138.4	100.7	4.9	20.7	11.6	7.87	37.0	1710	0.628	8.8	9.82	305.0	9.8	159.6	1.90	
26	68,901								2650	0.633	10.5	9.31	457.5	6.0	35.2	1.904	Inulin clearance 112 cc./min.
27	69,010								2030	0.652	6.5	8.12	454.5	3.5	78.3	1.904	
28	68,995	139.2	101.3	5.0	26.1	11.4	7.71	37.4	490	0.644	17.6	8.99	61.1	3.0	9.2	1.23	
29	68,650	137.9	101.2			12.8	7.42	38.3	870	0.635	27.9	10.23	66.3		20.4	1.23	
30	68,230																

Dietary intake was constant daily and consisted of: calories 2416, nitrogen 11.24 gms., sodium 8.8 mEq., chloride 22.8 mEq. and potassium 94.0 mEq.

TABLE III
Subject E. IV., ♂ age 22

Fast day	Serum and blood						Urine						Stool					Remarks						
	Na	Cl	K	CO ₂	BUN	Creat.	Prot.	Hemat.	Vol.	Creat.	NH ₄	Total N	Urea N	Uric acid	Na	Cl	K		P	Na	K	Cl	N	P
mf. 7.	per L.	per L.	mM/l.	mg%. %	mg%. %	gm%. %	gm%. %	% cells	cc.	gm%. %	mEq.	gm%. %	gm%. %	gm%. %	mEq.	mEq.	mEq.	mEq.	mEq.	mEq.	mEq.	gm%. %	mM	
1	141.2	101.2			15.2	1.0	7.70		1210	1.65	35.5	13.30	12.6		93.5	113.6	101.2	37.9						
2	141.2	101.2			15.2	1.0	7.70		1635	1.73	31.0	14.87	13.6		58.3	72.8	101.0	33.6						
3	141.2	101.2			15.2	1.0	7.70		1085	1.73	35.5	13.83	12.7		22.1	27.2	81.1	30.1						
4	141.2	101.2			15.2	1.0	7.70		770	1.81	32.1	14.18	13.0	0.792	8.1	22.9	81.6	35.1						
5	141.2	101.2	3.89		15.3	1.15		51.7	815	1.80	43.8	14.67	12.9	0.780	2.0	15.5	88.7	35.1	0.25	10.8	0.82	1.46	12.1	
6	141.2	101.2			15.2	1.10		52.0	795	1.83	39.7	15.48	14.9	0.824	1.9	15.5	88.7	31.2	0.25	10.8	0.82	1.46	12.1	
7	141.2	101.2			15.2	1.10		52.0	1410	1.71	31.4	14.69	13.7	0.733	1.3	8.7	103.2	36.5	0.25	10.8	0.82	1.46	12.1	
8	141.2	101.2			15.2	1.10		52.0	910	1.81	35.2	13.58	12.0	0.695	0.56	7.6	77.1	32.6	0.25	10.8	0.82	1.46	12.1	
9	141.2	101.2			15.6	1.15		7.27	1740	1.81	41.4	13.77	12.6	0.761	72.5	115.2	99.2	28.2	0.21	15.0	1.03	2.07	18.0	
10	141.2	101.2			15.6	1.15		7.27	2240	1.82	40.7	12.38	11.0	0.762	361.6	395.0	102.2	27.6	0.24	15.0	1.03	2.07	18.0	
11	141.2	101.2			15.6	1.15		7.27	2500	1.82	48.0	10.15	9.19	0.686	477.7	497.0	62.5	23.6	0.24	15.0	1.03	2.07	18.0	
12	141.2	101.2			15.6	1.15		7.27	2500	1.81	32.4	9.51	8.50	0.591	254.0	238.5	47.1	21.2	0.21	15.0	1.03	2.07	18.0	
13	141.2	101.2			15.6	1.15		7.27	1200	1.82	29.6	9.83	8.76	0.584	108.9	94.3	42.8	21.2	0.21	15.0	1.03	2.07	18.0	
14	141.2	101.2			15.6	1.15		7.27	1100	1.79	28.4	10.59	9.41	0.681	86.0	87.4	69.3	21.2	0.21	15.0	1.03	2.07	18.0	
15	141.2	101.2			15.6	1.15		7.27	1610	1.66	28.2	11.57	10.4	0.719	31.6	55.3	99.3	27.9	0.21	15.0	1.03	2.07	18.0	
16	141.2	101.2			15.6	1.15		7.27	951	1.71	26.4	11.06	10.0	0.667	3.72	25.1	91.3	27.7	0.10	10.8	0.34	1.59	13.4	
17	141.2	101.2			15.6	1.15		7.27	1060	1.76	35.6	14.79	13.5	0.774	1.78	20.7	103.3	30.8	0.10	10.8	0.34	1.59	13.4	
18	141.2	101.2			15.6	1.15		7.27	1000	1.72	33.0	11.69	10.3	0.661	2.52	17.1	83.4	21.0	0.10	10.8	0.34	1.59	13.4	
19	141.2	101.2			16.2	1.12		46.0	1555	1.80	3.9	12.20	11.5	0.768	214.6	2.8	217.7	36.2	1.01	10.4	0.58	2.27	20.5	
20	141.2	101.2			16.2	1.12		46.0	2170	1.80	4.8	10.96	9.97	0.747	374.7	5.4	101.9	36.8	1.01	10.4	0.58	2.27	20.5	
21	141.2	101.2			16.2	1.12		46.0	1620	1.67	2.6	9.31	8.34	0.689	434.6	4.1	58.3	28.8	1.01	10.4	0.58	2.27	20.5	
22	141.2	101.2			16.2	1.06		46.0	1590	1.75	10.6	9.30	8.14	0.609	188.2	6.7	22.0	25.4	0.97	7.25	0.88	2.00	17.3	
23	141.2	101.2			16.2	1.06		46.0	1125	1.73	14.5	9.52	8.27	0.584	71.4	8.3	32.2	20.6	0.97	7.25	0.88	2.00	17.3	
24	141.2	101.2			16.2	1.06		46.0	965	1.69	21.2	10.92	9.78	0.617	25.9	8.6	52.7	21.3	0.97	7.25	0.88	2.00	17.3	
25	141.2	101.2			16.2	1.06		46.0	1240	1.73	21.5	11.91	10.68	0.690	15.0	9.4	75.4	28.1	0.97	7.25	0.88	2.00	17.3	
26	141.2	101.2			16.2	1.06		46.0	1000	1.75	16.8	11.72	10.6	0.656	2.17	12.2	89.8	26.6	0.97	7.25	0.88	2.00	17.3	
27	141.2	101.2			16.2	1.06		46.0	817	1.85	25.2	13.35	12.2	0.727	1.78	15.4	106.5	33.1	0.97	7.25	0.88	2.00	17.3	
28	141.2	101.2			16.2	1.06		46.0	1060	1.82	21.0	11.59	10.9	0.656	7.54	19.8	100.5	28.2	0.97	7.25	0.88	2.00	17.3	
29	141.2	101.2			16.2	1.10		46.5	1000	1.71	31.7	12.26	11.2	0.695	2.96	21.3	71.8	29.9	0.97	7.25	0.88	2.00	17.3	
30	141.2	101.2			16.2	1.10		46.5	910	1.71	30.7	11.88	10.6	0.672	2.48	14.7	73.9	30.7	0.97	7.25	0.88	2.00	17.3	
31	141.2	101.2			16.2	1.15		48.0	910	1.71	32.2	11.01	10.3	0.679	5.18	17.2	76.6	26.6	0.97	7.25	0.88	2.00	17.3	
32	141.2	101.2			16.2	1.15		48.0	970	1.71	27.6	11.31	10.3	0.676	7.01	19.3	79.5	27.6	0.97	7.25	0.88	2.00	17.3	
33	141.2	101.2			16.2	1.15		48.0	920	1.75	32.6	10.99	9.96	0.724	2.72	17.5	69.5	29.6	0.97	7.25	0.88	2.00	17.3	
34	141.2	101.2			16.2	1.15		48.0	890	1.83	33.4	13.09	11.7	0.737	2.56	21.0	103.9	36.9	0.97	7.25	0.88	2.00	17.3	
35	141.2	101.2			16.2	1.15		48.0	1120	1.17	24.9	10.77	9.74	0.690	6.27	18.7	81.8	28.3	0.97	7.25	0.88	2.00	17.3	
36	141.2	101.2			16.2	1.15		48.0																
37	141.2	101.2			16.2	1.15		48.0																
38	141.2	101.2			16.2	1.15		48.0																
39	141.2	101.2			16.2	1.15		48.0																
40	141.2	101.2			16.2	1.15		48.0																
41	141.2	101.2			16.2	1.15		48.0																
42	141.2	101.2			16.2	1.15		48.0																
43	141.2	101.2			16.2	1.15		48.0																
44	141.2	101.2			16.2	1.15		48.0																
45	141.2	101.2			16.2	1.15		48.0																
46	141.2	101.2			16.2	1.15		48.0																
47	141.2	101.2			16.2	1.15		48.0																
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66	141.2	101.2			16.2	1.15		48.0																
67	141.2	101.2			16.2	1.15		48.0																
68	141.2	101.2			16.2	1.15		48.0																
69	141.2	101.2			16.2	1.15		48.0																
70	141.2	101.2			16.2	1.15		48.0																
71	141.2	101.2			16.2	1.15		48.0																
72	141.2	101.2			16.2	1.15		48.0																

Dietary intake was constant daily and consisted of: calories 3225, nitrogen 15.0 gms., sodium 9.4 mEq., chloride 24.9 mEq., potassium 100.1 mEq., and phosphorus 19.8 mM.

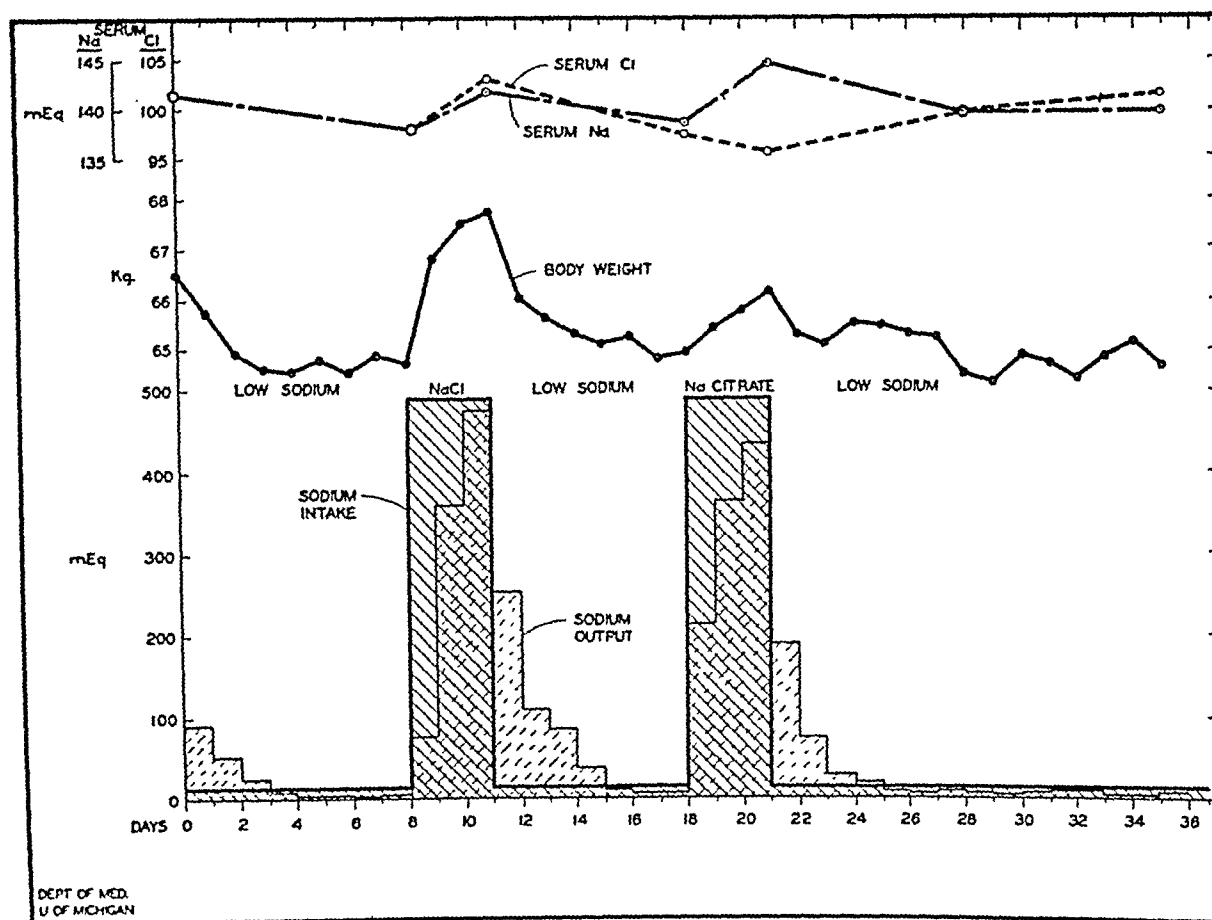


FIG. 1. To SHOW CHANGES IN BODY WEIGHT, SERUM SODIUM AND CHLORIDE CONCENTRATIONS, AND SODIUM BALANCE IN SUBJECT E. W.

changes were noted in extracellular fluid composition. The sodium restriction uniformly caused a small but significant lowering of serum sodium concentration and elevations occurred on administration of sodium. Serum chloride values followed a similar trend though less uniformly. Administration of the sodium citrate caused a rise in serum sodium in each case, a decrease in serum chloride in two cases and a rise in serum CO_2 content. A parallel rise in serum sodium and chloride followed sodium chloride administration. With the sodium citrate administration urinary sodium excretion rose sharply to high levels but urinary chloride loss was, if anything, further decreased. This is additional evidence that the kidneys can deal separately with these two most important ions of the extracellular fluid.

There is slight variability in stool sodium and chloride excretion. But considering the drastic changes in oral sodium and chloride intake from about 10 to 500 mEq. daily the stool losses remained surprisingly fixed and insignificantly small.

Fecal potassium excretion also showed no noteworthy variation.

Subject W. C. had one loose stool associated with abdominal cramps on the last day of sodium chloride administration. This one stool was collected and analyzed separately. Its sodium and chloride content was much higher than the remaining stools of that period.

Of greatest importance to this discussion are the observed changes in protein metabolism that occurred with variation of sodium intake. Figure 2 shows graphically the changes in nitrogen balance, urine urea excretion, blood urea nitrogen concentration, urine total uric acid excretion, urine potassium and phosphorus excretions in subject E. W. Exactly similar changes were observed in our other two subjects but because data are more complete in our last study on E. W. his results have been graphed. Sodium restriction is shown to produce slightly negative nitrogen balance during the first low sodium period. Following both periods of sodium administration the urine plus fecal nitrogen is considerably

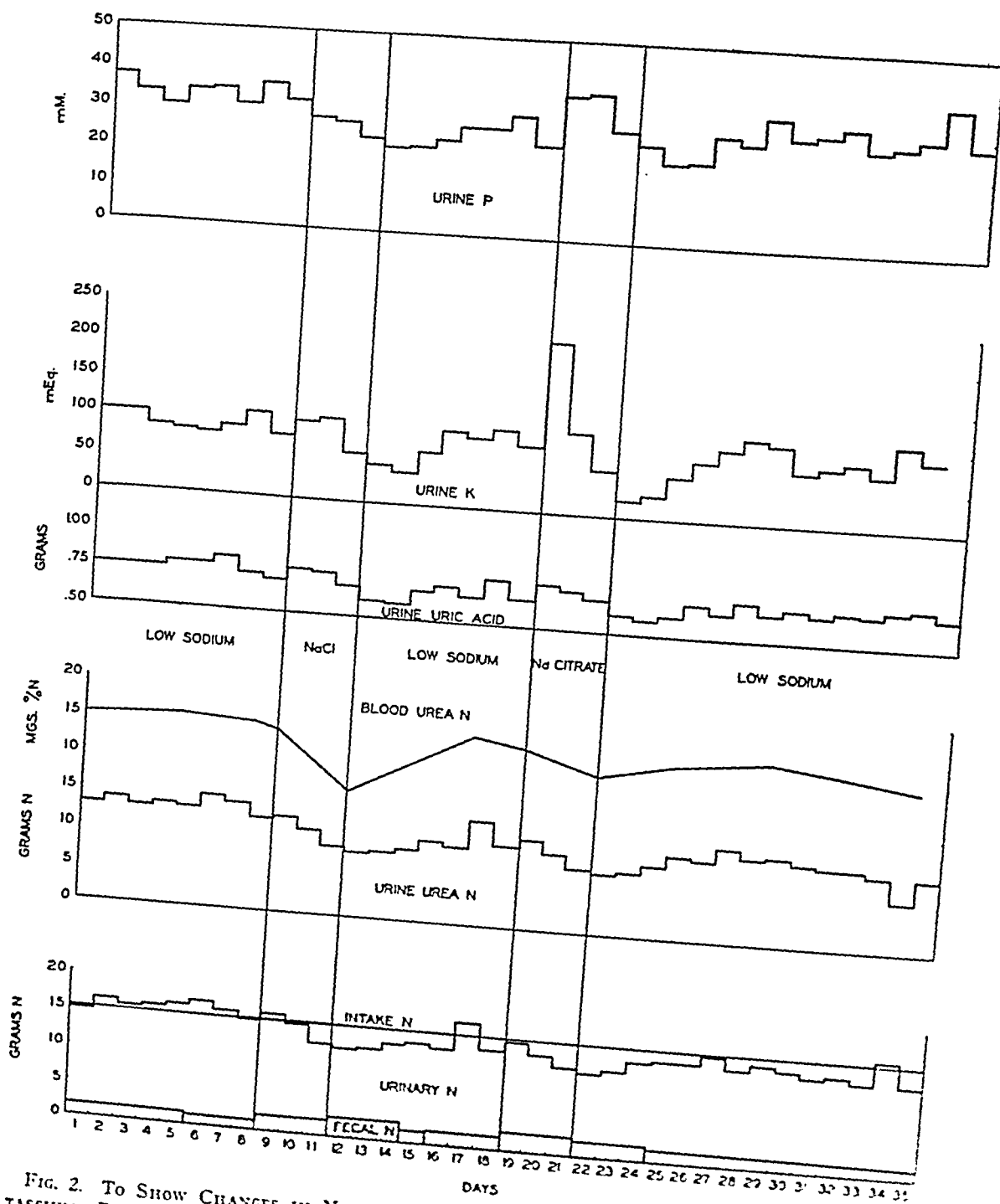


FIG. 2. TO SHOW CHANGES IN NITROGEN BALANCE, 24 HOUR URINARY UREA, URIC ACID, POTASSIUM, PHOSPHORUS EXCRETIONS AND BLOOD UREA NITROGEN CONCENTRATIONS IN SUBJECT E. W.

less than the intake of nitrogen so that nitrogen is stored in the body. The fecal nitrogen reveals that this decreased urinary nitrogen excretion was not the result of impaired nitrogen absorption from the alimentary tract.

Creatinine and ammonia excretion remained essentially constant so that the changes in urinary nitrogen excretion could be expected to be the result of changes in "metabolic" nitrogen. The urine urea excretion is seen to confirm this expectation since all changes in total urinary nitrogen are due almost entirely to changes in urine urea nitrogen (the difference between total urine nitrogen and urine urea nitrogen remaining very constant throughout the study).

That the changes in total and urea nitrogen excretion are not simply the result of storage of urea in the body fluids is indicated by the dramatic changes seen in the blood urea nitrogen. At the time when the urinary excretion of urea is at its lowest the blood urea concentrations are likewise lowest. Correcting the blood urea concentration for the dilution that might occur as the result of the increased body fluid volume produced by the sodium administration causes only insignificant changes. The serum creatinine was measured in the subject E. W. and though it shows minor fluctuations it in no way parallels changes in blood urea level. It has been amply proven that the serum creatinine concentration is independent of the level of protein catabolism (26). As there has occurred no impairment of gastrointestinal absorption of nitrogen these changes can be interpreted only as the result of a decreased rate of urea production or a decreased catabolism of protein associated with administration of sodium to individuals previously maintained on low sodium diets. These effects on protein metabolism occur following both sodium citrate and sodium chloride administration and therefore are the result of the sodium rather than of the accompanying anion.

Urine uric acid excretion, urine potassium and phosphorus excretion mimic the changes in nitrogen metabolism exactly except for the very high rate of potassium excretion on the first day of sodium citrate administration. This change is believed to be due to effects other than those on protein metabolism (6). Serum potassium concentrations failed to show any evidence of increasing

with the periods of low urine potassium excretion though unfortunately no serum potassiums were taken on the third or fourth days after the high sodium periods when this effect might have been present.

A point to be stressed is that there occurred a definite lag in the renal excretion of sodium during each high sodium period. Not until the third and last day of each high sodium period did the urinary excretion reach the level of the intake. Our interpretation of this lag is discussed below but here it should be noted that all the effects associated with the depressed protein catabolism were most pronounced immediately following rather than during the periods of high sodium administration, thus paralleling in this respect the lag in sodium excretion.

Urines were tested daily for reducing substance by the qualitative Benedict's test on subjects M. L. and E. W. but no evidence of glycosuria was noted. Glucose tolerance tests were done on subjects M. L. and E. W. M. L. showed no evidence of impaired glucose tolerance on the 11th day of the low sodium intake. (Standard glucose tolerance test after 1.75 gms. of glucose per kilogram of body weight revealed: fasting blood sugar—75 mgs.%; one hour—71 mgs.%; two hours—82 mgs.%; three hours—51 mgs.%; four hours—56 mgs.%.) E. W. on the 12th day of the final low sodium intake period showed: fasting blood sugar—89 mgs.%; after one hour—160 mgs.%; second hour—100 mgs.%; third hour—56 mgs.%. A repeat glucose tolerance test on E. W. two months after completion of this study and while on a normal dietary sodium intake revealed: fasting blood sugar—81 mgs.%; after one hour—153 mgs.%; after two hours—100 mgs.%; and after three hours—64 mgs.%.

DISCUSSION

Changes in nitrogen metabolism similar to those reported here have been noted by others. In studying experimental sodium deficiency produced by a low sodium intake and sweating in normal subjects McCance (27) found that his subjects developed negative nitrogen balances with rise in blood urea concentration. This observation was striking enough to be emphasized by the author. In a subsequent publication, McCance and Widdowson (28) conclude that the main cause

of this elevation of blood urea concentration with sodium depletion is the associated reduction in glomerular filtration rate. In another study McCance (29) observed that the administration of large doses of sodium chloride and sodium bicarbonate depressed the serum urea concentration significantly in previously dehydrated subjects. The electrolyte balance study of Albright and Bauer (30) on an edematous nephritic likewise shows an effect of administered sodium chloride and sodium bicarbonate on the nitrogen balance. Both sodium salts produced positive nitrogen balance. Landis *et al.* (31) reported striking changes in blood urea nitrogen levels in three subjects with varying degrees of renal insufficiency when the daily amount of sodium chloride intake was altered. Sodium chloride restriction caused increased blood urea nitrogen concentrations while administration of this salt lowered the concentration. These workers did 24 hour urea and creatinine clearances finding some increase in these clearances during the periods of sodium chloride administration. The decrease in blood urea nitrogen with administration of sodium chloride was associated with the increased renal clearances.

It is to be expected that administration of sodium chloride will increase glomerular filtration rate and hence urea clearance. Contrariwise, restriction of sodium intake in non-edematous patients with renal insufficiency is likely to produce severe dehydration through excessive renal loss of sodium with shrinkage of extracellular fluid volume. Hence, the increase in glomerular filtration rate with sodium administration is especially to be anticipated in patients with renal insufficiency (as were those of Landis *et al.*) who may be dehydrated prior to administration of the sodium.

The gain in weight observed in our subjects when sodium chloride was administered was almost entirely the result of an increase in extracellular fluid volume (as calculated from the gain in "chloride space" from the observed chloride balance). In such acute experiments as ours, changes in the hematocrit reflect changes in intravascular volume. Table IV clearly shows that sodium chloride administration results in a decrease in cell hematocrit with hemodilution. This increased intravascular volume may be expected, other factors remaining constant, to increase renal plasma flow with resulting increased glomerular

TABLE IV
E. W.
Hemodilution with increased rate of glomerular filtration following sodium chloride but not sodium citrate

Day	Hematocrit	Total serum protein	24 hour creatinine clearance	24 hour urea clearance
	% cells	gms. %	liters	liters
1		7.70	165	83
5	52		157	84
8	52	7.42	165	79
9		7.27	160	86
Period of sodium chloride administration				
12	43	6.38	192	128
15	48	7.19	133	90
17	50		153	92
19	46	6.91	161	81
Period of sodium citrate administration				
22	44	6.50	161	76
25	48	7.38	160	85
29	47	7.19	155	85
32	48	7.10	149	82

filtration rate. Another aspect of the hemodilution is the decrease in total serum protein concentration also shown in Table IV. This decrease would cause a decreased colloid osmotic pressure of the serum which also, other factors remaining constant, would favor an increased rate of glomerular filtration. The creatinine clearance figures in Table IV taken as a measure of glomerular filtration rate (32, 33) in E. W. show this expected increase when sodium chloride is administered. An even proportionately greater increase in the 24 hour urea clearance is seen. This indicates a reduced rate of tubular reabsorption of urea in addition to the increase in glomerular filtration rate. This is to be expected with the greater rate of urine formation that followed the sodium chloride administration. Inulin clearance measurements of glomerular filtration rate in subject W. C. on the low sodium intake and immediately following the high sodium chloride period reveal exactly similar changes in glomerular filtration rate (117 cc./min. after 12 days of low sodium intake and 158 cc./min. the day after the sodium chloride administration) with the same evidence of increased plasma volume and hemodilution. Female subject M. L. however showed no significant change in inulin clearance (121 cc./min. on the ninth day of low sodium intake or 123

cc./min. and 112 cc./min. on the days following the sodium chloride and sodium citrate period respectively). She also showed much less evidence of hemodilution than did the other two subjects. Sodium citrate produced no noteworthy changes in renal clearances in any subject.

Thus sodium chloride administration may to some degree lower the blood urea concentration by producing increased renal clearances in some cases. In our normal subjects the effect of altered renal dynamics on the blood urea concentration was, however, of lesser importance. In spite of the increased urea clearance in subjects E. W. and W. C. following sodium chloride, the 24 hour urea excretion was markedly reduced. The only way an increased clearance alone can decrease blood concentrations of a substance is by an increased rate of removal of that substance from the body fluids. But our data in all cases reveal a sharp decrease in the rate of urea and nitrogen excretion just at the time of lowest blood concentrations. Sodium citrate, which failed to increase the glomerular filtration rate, also produced a depression in blood urea concentration. In subject M. L. no noticeable increase in glomerular filtration rate was apparent on the day following administration of sodium chloride. However, the blood urea nitrogen depression still occurred.

In the presence of normal alimentary absorption of nitrogen and decreased renal excretion of it, the drop in blood concentrations of urea in our subjects must have been the result of a decreased rate of catabolism of protein. This accounted for the positive nitrogen balance resulting from administration of sodium. Figure 2 clearly shows that such an interpretation is justified.

The purpose of this study was to learn whether the physiological regulation of body sodium content is controlled by adrenal cortical activity in normal persons who ingest variable amounts of sodium. It has been amply demonstrated that the adrenal cortex produces hormones which are capable of decreasing renal sodium excretion (1, 5, 34) and increasing catabolism of protein (35, 36). The former has a desoxycorticosterone-like action and has been termed the "salt-and-water" hormone (or hormones) while the latter is protein catabolic or anti-anabolic and will be called the "protein catabolic" hormone in this discussion.

This does not prove, however, that in the normal subject the desoxycorticosterone-like hormone actually controls sodium balance. But if these two apparently unrelated effects of adrenal cortical activity (*i.e.*, on sodium and on protein) are elicited by the single stimulus to conserve body sodium, this will afford good evidence that the adrenal cortex is importantly involved in regulation of normal body sodium.

With these two actions of the adrenal cortex in mind, the changes noted in Figure 2 lend themselves readily to explanation on the basis of alterations of adrenal cortical activity as follows. In the first eight days of sodium restriction renal excretion of sodium decreased to a minimum under the stimulus of increased adrenal salt-and-water hormone activity. At the same time the rate of protein catabolism resulting from increased protein catabolic hormone activity caused a negative nitrogen balance with the levels of excretion of urea, uric acid and potassium as charted. Administration of large amounts of sodium chloride on the ninth, tenth and 11th days removed the requirement for renal conservation of sodium. The adrenal responded by decreasing the activity of its salt-and-water hormone output allowing the kidneys to increase their rate of sodium excretion enormously. At the same time the protein catabolic hormone was likewise depressed with decreased protein catabolism, a positive nitrogen balance, decreased uric acid, urea, potassium and phosphorus excretions. Further sodium restriction from the 12th to 19th day resulted again in a decreased urine sodium excretion (increased adrenal activity). The following three day period of sodium citrate administration reproduces the same effects of decreased adrenal cortical function that occurred with sodium chloride administration, followed finally by increased adrenal cortical activity when sodium intake was again restricted during the final 14 days of the study.

Figure 3 was constructed from the observed potassium and phosphorus balances of subject E. W. The theoretical potassium and phosphorus balances were calculated from the observed nitrogen balance. It is noted from this figure that the decreased potassium excretion that followed each period of sodium administration can only partially be accounted for by the decreased catabolism of protein that occurred at that time. Knowing that

desoxycorticosterone, the salt-and-water hormone par excellence, produces an increased renal excretion of potassium (37, 38) while patients with Addison's disease and adrenalectomized animals have a decreased ability to excrete potassium (1, 39), this observed drop in potassium excretion is taken to be, at least in part, the result of a decreased salt-and-water hormone activity occurring at this time when there is a large renal sodium ex-

cretion. Unfortunately, the serum potassium concentration was not determined immediately following the days of lowest urinary potassium excretion to ascertain whether or not an elevation of serum potassium occurred as a result of this decreased renal potassium excretion. The strikingly negative potassium balance on the first day of sodium citrate administration is discussed in another paper (6).

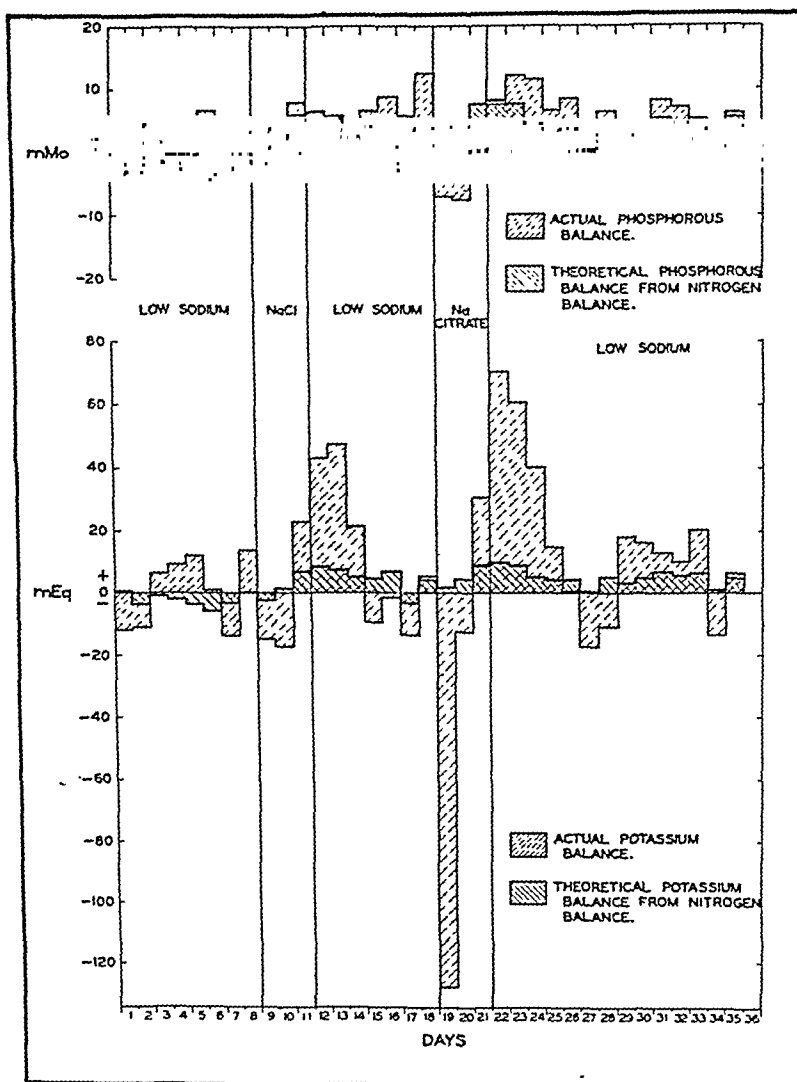


FIG. 3. THE ACTUAL AND THEORETICAL POTASSIUM (LOWER) AND PHOSPHORUS (UPPER) BALANCES OCCURRING IN SUBJECT E. W.

Output values were determined from the sum of urinary and fecal contents and intake values were obtained from diet analysis. The factor 2.7 was used to convert grams of observed nitrogen balance to milli-equivalents of "theoretical potassium balance" and the factor 2.15 to convert grams of observed nitrogen balance into millimols of "theoretical phosphorous balance." Note especially the large positive actual potassium balance immediately following both periods of sodium administration.

A comparison of the phosphorus and nitrogen balances, also illustrated in Figure 3, reveals no significant differences between actual and theoretical phosphorus balance. The theoretical phosphorus balance was not, however, corrected for possible changes in bone metabolism.

Our data show more distinct evidence of depression of adrenal cortical activity with administration of sodium than stimulation of activity by restriction of sodium. However, with more severe sodium depletion produced by sweating in addition to very low sodium dietary intake, one of McCance's subjects had a negative nitrogen balance of 45 gms. in 11 days of sodium depletion and McCance states (27) "... this illustrated rather well the fact that the nitrogen balance steadily became more negative as sodium chloride was withdrawn from the body." Conn (40) found that sodium restriction in normal subjects undergoing acclimatization to humid heat results in negative nitrogen balance. This investigator demonstrated that it was the sodium restriction rather than a nonspecific effect of the hot environment which caused the negative nitrogen balance. Thus when acclimatization to the hot environment was complete in his subjects while they were on a fixed sodium intake, nitrogen balance was regained. Further restriction of sodium, without changing the exposure to the hot environment or otherwise altering the diet, was again followed by a negative nitrogen balance, which disappeared gradually as reacclimatization was accomplished on the lower sodium intake. Thus, it seems that the amount of evidence of increased protein catabolic hormone activity that is elicited in response to sodium restriction is dependent upon the intensity of the requirement to conserve body sodium.

It is also evident from our data that with prolongation of the period of low sodium intake the effects on nitrogen metabolism attributed to adrenal activity (protein catabolic hormone action) disappear. This occurs in spite of the continued renal conservation of sodium. In all three subjects the protein catabolic effects were most evident early in the study. As mentioned above, Conn (40) found that during acclimatization to a hot environment negative nitrogen balance occurs early and gradually disappears. It is of interest in this connection to note that Swanson and Smith

(41) found that rats kept on a "salt-free" diet showed a definite increase in weight of the adrenals followed by regression, with adrenal weights after 90 days only slightly greater than in the control rats receiving salt.

Apparently then, with continuation of the requirement to conserve body sodium, the protein catabolic hormone effect disappears. This could be the result of a decreased adrenal cortical output of protein catabolic hormones or of a decreased response on the part of the tissues to the action of an increased protein catabolic hormone in the body. Our data give no clue as to whether one of the two or both possibilities actually occurs.

A further point of interest should be mentioned. That is the definite lag that occurred in the renal excretion of sodium when it was administered to our subjects following the low sodium intake, and the lag in the renal conservation of sodium that occurred when the sodium intake was again sharply reduced. Because the changes noted in nitrogen balance and renal potassium excretion with sodium administration show exactly the same lag, it appears that the sudden changes in sodium intake require some time to produce an adrenal response.

We are well aware that this interpretation of our results suffers considerably from the lack of adrenal cortical hormone assays which would more directly define the role of the adrenal cortex.³ However at the present time there is no method of measuring directly adrenal salt-and-water hormone production. Forsham *et al.* have found increased uric acid excretion to be a more consistent and more marked result of prolonged administration of 11 and 11-17 oxycorticosteroids than is the nitrogen balance (42). Further studies by this group of uric acid excretion in response to adrenocorticotrophin administration (43) support the view that uric acid excretion reflects changes in 11-oxycorticosteroid activity. The observed changes in total uric acid excretion in subject E. W. on a constant purine diet (see Figure 2) clearly then support our interpretation of altered adrenal cortical activity in response to varying sodium intake.

³ Space does not permit a discussion of possible changes in androgenic and protein anabolic hormone activity of the adrenal cortex and our data show no positive evidence that such changes occurred.

Because evidence of both protein catabolic hormone activity and of salt-and-water hormone activity has been elicited by the need to conserve body sodium it is suggested that the adrenal received its direct stimulus via the anterior pituitary. Studies with adrenocorticotrophic hormone administration to human beings support this view since this hormone causes stimulation of protein catabolic effects as well as salt-and-water hormone effects (40, 43).

The fact that the changes observed were similar following both sodium chloride and sodium citrate administration indicates that the sodium cation is of importance rather than the anion. Just what change occurs in the body when sodium intake is restricted or high, which serves to initiate the adrenal cortical activity, is not revealed. Changes in concentration of extracellular fluid, sodium, chloride, CO_2 content, tonicity of extracellular fluid or volume of extracellular fluid perhaps through its effect on circulating intravascular volume, all suggest themselves, but a multitude of other changes which occur in the extra- and intracellular fluids with variation in sodium intake might as likely act as the actual stimulus. The work of Selye on the general adaptation syndrome (44) suggests that our observations might simply cover an adaptation to sodium restriction as the stress, but this would only further describe rather than explain our results. Our findings and interpretation are in agreement with those of Conn (40), who demonstrated an increased adrenal cortical activity in response to the requirement to conserve salt during acclimatization to humid heat.

SUMMARY AND CONCLUSIONS

Some effects of marked variation in sodium intake in three normal young adult subjects were studied. These subjects were maintained on fixed diets containing adequate calories and protein with very low sodium and chloride content. At definite periods the sodium intake was abruptly increased by the addition of 480 mEq. of sodium as sodium chloride or sodium citrate.

With reduction from usual intakes of sodium to 9 mEq. of it daily there occurred an initial negative balance of sodium and chloride followed by progressive reduction of urinary losses to minimal amounts in about five days. This initial loss of body sodium and chloride was associated with a

loss of about 2 kilograms of weight with subsequent stabilization of weight as urine sodium and chloride loss became very small. Slight decrease in serum sodium and chloride levels also occurred.

Administration of sodium as sodium chloride or as sodium citrate was accompanied by a sharp increase in body weight much more pronounced with sodium chloride than with the citrate. There also occurred a hemodilution as evidenced by decrease in serum proteins and hematocrit and an increased glomerular filtration rate. These latter effects were much more pronounced after sodium chloride administration than after sodium citrate.

A definite lag in the renal excretion of sodium with the shift from the low to the high sodium intake and a delay in the renal conservation of sodium with change from the high to the low sodium intake occurred.

With this variation in sodium intake definite changes in protein metabolism occurred. Administration of sodium after sodium restriction produced positive nitrogen balance while sodium restriction increased the rate of protein catabolism. These changes were reflected in increased 24 hour urea, uric acid, phosphorus and potassium excretions with sodium restriction and depression of these urinary excretions with sodium administration. In addition a definite decrease in blood urea concentration occurred with each period of sodium administration which could not be accounted for by alteration in renal clearance of urea but was the result primarily of a decrease in the rate of protein catabolism.

The decreased urinary potassium excretion following the periods of sodium administration could not be entirely accounted for by the decrease in protein catabolism that also occurred at this time and this effect is discussed.

We believe that all the observed effects are best explained by changes in adrenal cortical activity. In this sense the requirement for conservation of body sodium in the presence of a very low sodium intake was met by increased adrenal cortical activity of a desoxycorticosterone-like hormone, accompanied by increase in activity of adrenal protein catabolic hormone. Administration of large quantities of sodium abolished this need to prevent renal loss of sodium with resultant decrease in adrenal cortical activity. Our findings indicate that in the normal subject, the conserva-

tion of body sodium by variation of renal excretion of sodium is the result of increased adrenal cortical activity in response to low sodium intake and decreased adrenal cortical activity when large amounts of sodium are administered.

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SOME EFFECTS OF VARIATION IN SODIUM INTAKE AND OF DIFFERENT SODIUM SALTS IN NORMAL SUBJECTS¹

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In a separate paper (1) we have described effects resulting from variation of sodium intake in normal subjects, which we attribute to changes in adrenal cortical activity. It is the purpose of this paper to describe some additional effects of considerable physiological interest that were observed in the course of that study.

METHODS

Three normal young adults were maintained throughout the study on a constant diet adequate in calories and protein with a very low sodium (about 9 mEq.) and chloride (about 25 mEq.) content. At definite periods 480 mEq. of sodium were administered orally daily either as sodium chloride or as sodium citrate. The analytical methods and diet are described in detail in the previous report (1).

RESULTS

The complete data of this study were presented in a separate paper (1). Only data pertinent to the present discussions will be presented here.

Table I shows the weights of our three normal subjects for the three days before, during and following sodium administration. It is evident that with sodium chloride administration a much more marked gain of weight occurred than with sodium citrate. Each subject was on a maintenance caloric intake so that practically all weight changes occurring over such short periods as these are due to change in body fluid content.

Table II shows the changes in urinary sodium, chloride and potassium excretion that occurred before, during and following each period of sodium administration. No significant changes in fecal sodium, chloride, and potassium were noted and these are not recorded here. It is seen that with administration of sodium chloride there is in each case a parallel increase in both urinary sodium and chloride as would be expected. Following the

sodium citrate administration a similar increase in sodium excretion is noted; however, chloride excretion remains unchanged, or if anything, slightly decreases.

Table II shows further that with the sodium chloride administration no significant increase in potassium excretion occurred except in the case of M. L., whose urine potassium content rose from about 40 mEq. in 24 hours to 88 mEq. the first day of sodium chloride administration. In subjects W. C. and E. W. a significant decrease in potassium excretion occurred after cessation of sodium chloride administration. On the first day of sodium citrate administration, a very definite increase in potassium excretion took place in all subjects. This was followed, after omission of sodium citrate, by a decrease to unusually low levels of potassium excretion.

Table III shows the data we have collected re-

TABLE I

Nude, fasting body weights of subjects for the three days before, the three days of sodium administration and the three following days

W. C.	M. L.	E. W.	
75.005	68.285	64.650	Low sodium and chloride
74.950	68.620	64.920	Low sodium and chloride
74.565	68.640	64.805	Low sodium and chloride
76.830	70.370	66.840	Sodium chloride 479 mEq.
78.250	71.480	67.500	Sodium chloride 479 mEq.
77.748	71.270	67.660	Sodium chloride 479 mEq.
74.770	70.020	66.070	Low sodium and chloride
74.480	69.860	65.700	Low sodium and chloride
74.100	69.480	65.400	Low sodium and chloride
75.750	68.650	65.230	Low sodium and chloride
75.660	68.670	64.820	Low sodium and chloride
74.850	68.475	64.990	Low sodium and chloride
75.370	68.901	65.440	Sodium citrate 478 mEq.
75.830	69.010	65.730	Sodium citrate 478 mEq.
75.850	68.995	66.080	Sodium citrate 478 mEq.
75.330	68.650	65.340	Low sodium and chloride
75.030	68.230	65.110	Low sodium and chloride
75.150		65.450	Low sodium and chloride

¹ This study was aided by a grant from the United States Public Health Service.

TABLE II

Urinary sodium, chloride and potassium excretions of subjects for three days before, during, and three days following each three day period of sodium administration

W. C.			M. L.			E. W.			
Na	Cl	K	Na	Cl	K	Na	Cl	K	
(mEq.)	(mEq.)	(mEq.)	(mEq.)	(mEq.)	(mEq.)	(mEq.)	(mEq.)	(mEq.)	
13.2	5.9	49.8	2.6	2.7	33.2	1.9	15.5	88.7	Low sodium and chloride
17.0	7.0	49.9	1.6	1.0	42.8	1.3	8.7	103.2	Low sodium and chloride
1.5	4.9	68.2	1.2	0.9	41.2	0.6	7.6	77.1	Low sodium and chloride
20.4	16.8	68.2	152.2	152.6	87.6	72.5	115.2	99.2	Sodium chloride 479 mEq.
250.2	267.2	81.0	371.2	411.2	76.0	361.6	395.0	102.2	Sodium chloride 479 mEq.
425.8	466.8	83.5	582.9	614.4	41.6	477.7	497.0	62.5	Sodium chloride 479 mEq.
292.9	300.7	78.4	201.9	188.4	30.4	254.0	238.5	47.1	Low sodium and chloride
53.4	57.2	33.8	53.5	62.8	67.6	108.9	94.3	42.8	Low sodium and chloride
70.5	63.2	34.5	31.0	42.8	66.8	86.0	87.4	69.0	Low sodium and chloride
4.2	14.4	71.8	6.4	8.4	66.4	3.72	25.1	91.3	Low sodium and chloride
5.4	11.2	79.7	5.7	7.8	60.4	1.78	20.7	103.3	Low sodium and chloride
7.1	16.8	84.4	4.5	9.2	66.4	2.52	17.1	83.4	Low sodium and chloride
284.7	13.2	147.6	305.0	9.8	159.6	214.6	2.8	217.7	Sodium citrate 478 mEq.
430.9	10.2	128.6	457.5	6.0	85.2	374.7	5.4	101.9	Sodium citrate 478 mEq.
439.0	12.0	66.6	454.5	3.5	78.8	434.6	4.1	58.3	Sodium citrate 478 mEq.
112.7	9.6	40.0	61.1	3.0	9.2	188.2	6.7	22.0	Low sodium and chloride
50.1	9.6	31.2	66.3		20.4	71.4	8.3	32.2	Low sodium and chloride
26.0	6.2	53.6				25.9	8.6	52.7	Low sodium and chloride

garding glomerular filtration rate and sodium and chloride excretion in these normal subjects. In subject E. W. the glomerular filtration rate is estimated from the 24 hour endogenous creatinine clearance (2, 3). In our other two subjects, glomerular filtration rate was determined by inulin clearances (4). The sodium and chloride excretions were obtained simultaneously with the filtration rates.

DISCUSSION

The discussion is organized about the four points we wish to stress:

I. The importance of fixed anion as well as cation to the accumulation of extracellular fluid.

As is well known, quantitatively the important cation of the extracellular fluids is sodium, and through renal regulation the concentration of sodium in the extracellular fluids is maintained within very narrow limits. Even in disease it is uncommon to find the serum sodium concentration varying more than 20 mEq./L from the normal value of about 140 mEq. Thus, it has come to be generally realized that extracellular fluid volume does not increase without available sodium

(5). This fact has become the central principle in the management of clinical edema by sodium restriction.

Furthermore it is true that the important anion of the extracellular fluid regarded quantitatively is chloride. Little, however, is mentioned regarding the role played by chloride in the accumulation of extracellular fluid. Table I and Figure 1, which show the changes in body weight that occurred with administration of equivalent amounts of sodium first as sodium chloride and then as sodium citrate, clearly indicate that weight gain is much greater following administration of sodium chloride. In short experiments of this type, change in weight is necessarily due almost entirely to changes in body fluid content. Calculation of predicted weight change from increase in chloride space following sodium chloride administration reveals that the weight gain is almost entirely due to increase in extracellular fluid. Figure 1 shows that the serum sodium and chloride concentrations in subject E. W. rose slightly and equally on administration of sodium chloride.

The inability to accumulate much extracellular fluid and gain weight when the same amount of sodium was administered as the citrate salt was

TABLE III

Simultaneous glomerular filtration rate and urine sodium and chloride excretions

	Experimental day	*Glomerular filtration	Urinary sodium	Urinary chloride	
		<i>L/24 hrs.*</i>	<i>mEq./24 hrs.</i>	<i>mEq./24 hrs.</i>	
Subject E. W.	1	165	94	114	<i>Low Na and Cl</i> Sodium—9.4 mEq. Chloride—24.9 mEq. Daily
	5	157	2.0	15.2	
	8	165	0.6	7.6	
	9	160	72.5	115	<i>High Na and Cl</i> Sodium—488 mEq. Chloride—504 mEq. Daily
	10				
	11				
	12	192	254	239	<i>Low Na and Cl</i> Sodium—9.4 mEq. Chloride—24.9 mEq. Daily
	15	133	31.6	55.3	
	17	153	1.8	20.7	
	19	161	215	2.8	<i>High Na citrate</i> Sodium—488 mEq. Chloride—24.9 mEq. Daily
	20				
	21				
	22	161	188	6.7	<i>Low Na and Cl</i> Sodium—9.4 mEq. Chloride—24.9 mEq. Daily
	25	160	15.0	9.4	
	29	155	3.0	13.3	
	32	149	7.0	19.3	
Subject W. C.	12 (first period)	<i>ml./min.**</i> 121	<i>mEq./min.</i> 0.020	<i>mEq./min.</i> 0.017	<i>Low Na and Cl</i> (as above)
	(second period)	113	0.018	0.011	
	25 (first period)	156	1.56	1.29	<i>High Na and Cl</i> (as above)
	(second period)	159	1.07	1.07	
Subject M. L.	9 (first period)	117	0.098	0.093	<i>Low Na and Cl</i> (as above)
	(second period)	124	0.17	0.16	
	17 (first period)	126	0.53	0.46	<i>High Na and Cl</i> (as above)
	(second period)	119	0.71	0.67	
	29 (first period)	113	0.081	0.024	<i>High Na citrate</i> (as above)
	(second period)	111	0.216	0.016	

* Glomerular filtration rate was determined as the 24 hour endogenous creatinine clearance in subject E. W.

** Glomerular filtration rate was determined as the inulin clearance in subjects W. C. and M. L.

apparently due to the lack of fixed anion, specifically chloride. Since the citrate anion is metabolized in the body it leaves the sodium cation without an accompanying fixed anion. Classical studies have shown that when sodium enters the extracellular fluid compartment without equivalent fixed anion it becomes largely associated with bicarbonate, equivalent for equivalent, keeping the total cation and anion concentrations of the extracellular fluids equal. Such an increase in bicarbonate concentration tends to produce an alkalosis (rise in pH above the normal value 7.35) in the extracellular fluids. The magnitude of this disturbance is diminished by removal of sodium

from the extracellular fluid by renal excretion and by its entry into the intracellular compartment. The extent to which these two regulatory mechanisms failed to prevent a temporary increase in bicarbonate concentration in the extracellular fluid of E. W. is indicated in Figure 1 by the rise in serum sodium from 138.6 to 144.9 mEq. per liter and drop in serum chloride from 97.6 to 95.6 mEq. per liter that occurred with the sodium citrate administration. The serum CO₂ content rose from 30.3 to 37.3 mM per liter. As the low chloride intake had already resulted in very small amounts of chloride in the urine, the kidneys were unable to make fixed anion available in significant amounts

by any further reduction in urine chloride excretion. The slight weight gain that occurred was associated with the small expansion of extracellular fluid volume made permissible by the dilution of the existing chloride content of the extracellular fluid. Thus in the absence of fixed anion, administration of sodium, even in large amounts, does not increase extracellular fluid volume importantly.

II. Dissociation of renal sodium and chloride excretion

Table II clearly shows that when sodium chloride is administered there occurs a parallel increase in urinary excretion of sodium and chloride. With the administration of sodium citrate a similar increase in urinary sodium occurs but the urinary chloride continues to be small or even

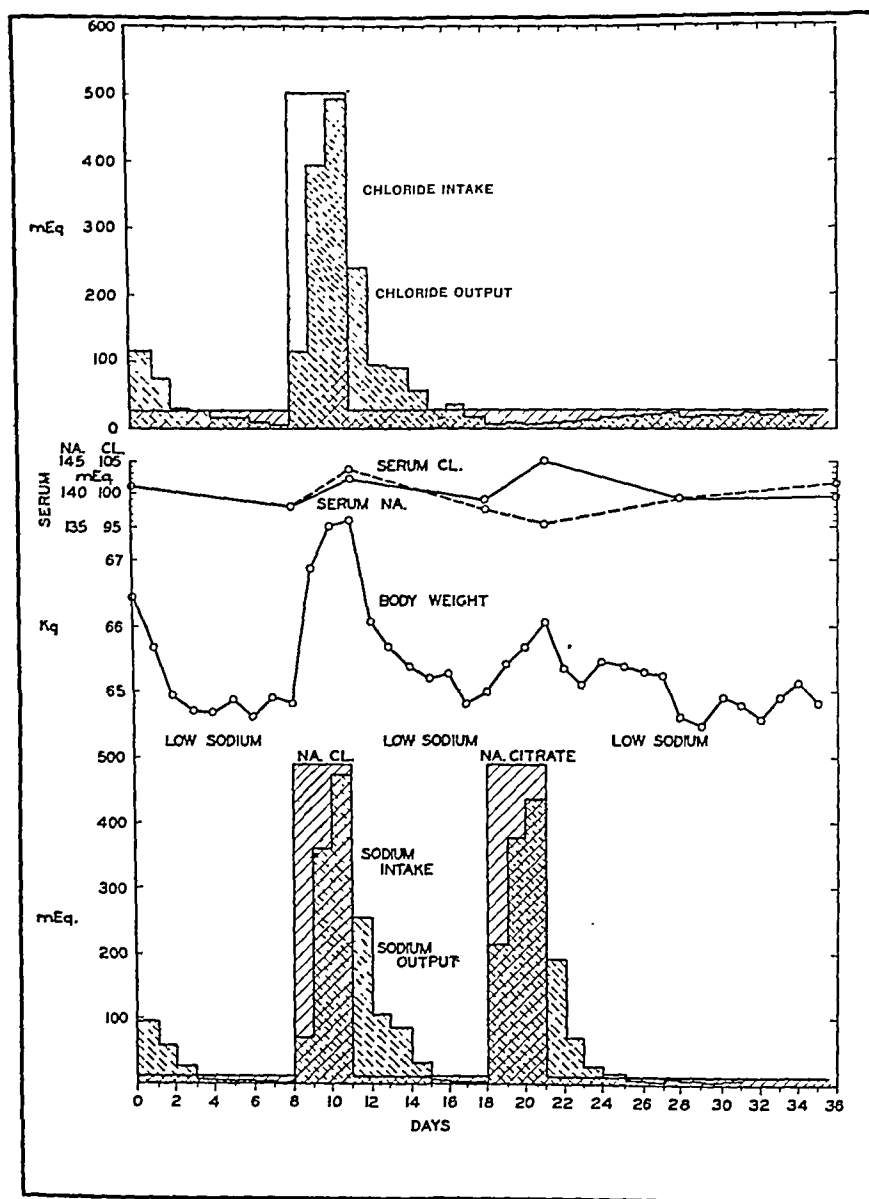


FIG. 1. TO SHOW CHANGES IN BODY WEIGHT, SERUM SODIUM AND CHLORIDE CONCENTRATIONS TOGETHER WITH SODIUM (LOWER) AND CHLORIDE (UPPER) BALANCES IN SUBJECT E. W.

Note especially the decreased chloride excretions occurring during the period of sodium citrate administration.

decreases below the base line. This is illustrated in Figure 1 for subject E. W.

In another report (1) we present evidence to support the belief that the renal excretion of sodium in normal human subjects is regulated by adrenal cortical activity. Earlier studies by others of the action of adrenal cortical extracts and adrenal cortical hormones on sodium and chloride balance suggested a similar effect on both sodium and chloride (6-8). Such studies in the past have been conducted under conditions that might be expected to cause parallel changes in sodium and chloride balance, *i.e.*, loss of sodium and chloride in the urine following adrenalectomy and retention of sodium and chloride on administration of adrenal cortical extract or adrenal cortical steroids to adrenalectomized animals. But when a definite need on the part of the body to conserve fixed anion, chiefly chloride, is created because of the presence of a large excess of sodium cation, a complete dissociation in the renal excretion of sodium and chloride is elicited. Our earlier studies (1) indicate that large intakes of sodium, either as the chloride or citrate salts, depress adrenal cortical activity. This would permit the large urinary excretion of sodium that followed the administration of sodium citrate; but accompanying this extensive removal of sodium there is no increased loss of chloride. In fact the urinary values for chloride fell below those of the control period. What it is that causes the kidneys to conserve chloride when excess base is administered, this study does not indicate. It is recognized, however, that such reduction of renal excretion of chloride serves a useful function in conserving fixed anion in the presence of the threat of alkalosis resulting from the sodium citrate administration—another example of the "wisdom" of the body.

III. Displacement of intracellular potassium by sodium

It was noted that each of our subjects showed a prompt and striking increase in urinary potassium excretion during the first 24 hours of sodium citrate administration. By the third day of the sodium citrate period the urinary potassium had dropped to or below the base line (see Table II). No such increase in urinary potassium content occurred with sodium chloride administration ex-

cept possibly for the much less striking increase in subject M. L. Figure 2 shows the theoretical potassium balance calculated from the actual nitrogen balance (grams nitrogen balance $\times 2.7 =$ mEq. theoretical potassium balance on the basis of changes in body protoplasm) and indicates that this potassium excretion was not the result of increased catabolism of tissues. Figure 2 also clearly shows that this marked potassium diuresis coincided with the markedly positive balance of sodium unaccompanied by fixed anion that resulted from the sodium citrate administration.

To learn more about the association of positive sodium and negative potassium balance the calculations shown in Table IV were made according to the method of Darrow (9) for the periods of sodium administration in each subject. The extracellular fluid volume was estimated as 20%² of the body weight on the morning of the first day of sodium administration. It is seen from Table IV that in each case with sodium citrate administration there is a positive intracellular sodium balance, definite in E. W. (222 mEq.) and M. L. (178 mEq.) and perhaps equivocal in subject W. C. (83 mEq.). This is associated with a negative

² Recent evidence by inulin space determinations (20) indicates that the extracellular fluid volume may be less than this per cent. Our subjects also had some shrinkage of extracellular volume as a result of the preceding restriction of sodium and chloride intake. However, the value taken for the initial extracellular fluid volume in these calculations, within reasonable limits, only insignificantly affects the results.

TABLE IV
*Changes in intracellular sodium and potassium balances that occurred during the three-day periods of sodium chloride and sodium citrate administration**

	Total Na balance	Total K balance	Extra-cell-Na balance	Extra-cell-K balance	Intra-cell-Na balance	Intra-cell-K balance
	mEq.	mEq.	mEq.	mEq.	mEq.	mEq.
<i>Subject E. W.</i>						
after NaCl	+553	-9	+644	+13	-91	-22
after NaCit.	+436	-108	+214	+2	+222	-110
<i>Subject M. L.</i>						
after NaCl	+355	+46	+401	+11	-46	+35
after NaCit.	+236	-76	+58	+3	+178	-79
<i>Subject W. C.</i>						
after NaCl	+759	-60	+722		+37	
after NaCit.	+303	-133	+220	0	+83	-133

* The complete data from which these calculated values were derived are presented in our preceding paper (1).

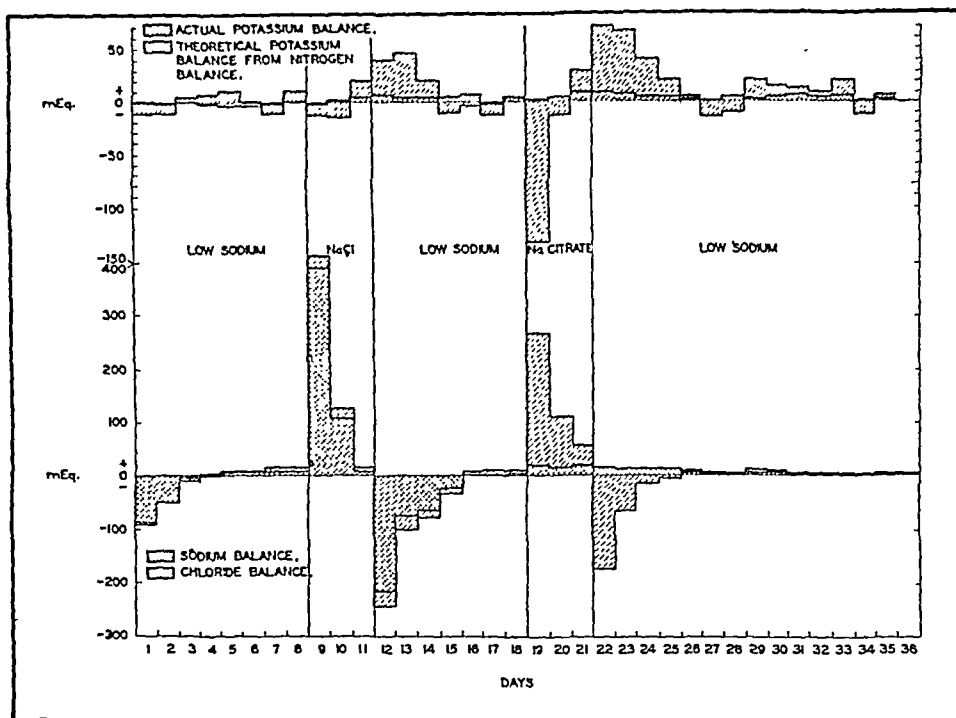


FIG. 2. THE ACTUAL AND THEORETICAL POTASSIUM BALANCES (UPPER) AND SODIUM AND CHLORIDE BALANCES (LOWER) IN SUBJECT E. W.

Note that the sodium and chloride balances change together except during and following the period of sodium citrate administration. The factor 2.7 was used to convert grams of observed nitrogen balance to milli-equivalents of "theoretical potassium balance." The values plotted are the difference between intake and urine plus fecal excretions. Note the large negative potassium balance during the first day of sodium citrate administration.

intracellular potassium balance of -110 , -79 , and -133 mEq. respectively for the three-day period.

This method of calculation originated by Darrow is based on the assumption that nearly all of the chloride of the body is restricted to the extracellular fluid compartment. The significant positive figures for "intracellular sodium balance" (or sodium that cannot be accounted for in the extracellular fluids of the body) clearly indicate that sodium has entered some part of the body unaccompanied by chloride. The coincident strongly negative potassium balance is good evidence that this sodium entered the intracellular compartment without chloride, displacing potassium, in so doing. We make no attempt to quantitate the intracellular sodium gain with the potassium loss, as extraneous factors, such as increase in tonicity of the extracellular fluid that occurred with the sodium administration and which might require changes in the total cation concentration within the cells, invalidate such attempts.

Administration of the sodium cation with fixed anion as sodium chloride resulted in no such gain in intracellular sodium. The total gain in body sodium was associated with nearly an equivalent gain of chloride. The sodium gain was accounted for by the increase in chloride space—expanded extracellular fluid volume. There was no occasion for sodium to pass into the cells. Hence no displacement of potassium occurred.

These findings, namely, displacement of intracellular potassium on administration of sodium citrate,³ strongly support the excellent studies of Darrow (8, 10-13) that the intracellular sodium plays a role in the defense of the normal extracellular pH.

The data presented in the preceding publication (1) offer an explanation of the lag in excretion of sodium on changing from low to high intake

³ One may anticipate exactly similar effects from sodium bicarbonate, acetate, lactate or other sodium salts having an associated anion that will be metabolized to bicarbonate in the body.

of sodium. Namely that the sharp restriction of sodium just before the administration of sodium citrate, resulted in maximal reabsorption of sodium in response to augmented adrenal cortical activity. This effect in decreasing amounts was continued for several days after the subjects had begun to take the sodium citrate. Thereafter, the adrenal drive relaxed and the large intake of sodium was accompanied by a plentiful excretion of sodium. During this lag in renal adjustment, the sodium cation passed into the cells of the body from the extracellular fluids, without accompanying fixed anion. This reduced the "available base" (or CO_2 content) of the extracellular fluids, and hence the extent of alkalosis that would have otherwise been produced by the sodium citrate administration. Had this shift of sodium into the cells failed to occur in subjects E. W. and M. L. an increase in the extracellular CO_2 content of 15 and 12 mM per liter, respectively, might have occurred with a resultant severe alkalosis.

The renal excretion of the potassium displaced from the cells was not impeded by the augmented adrenal cortical activity as was the sodium, since the potassium appeared in the urine. Desoxycorticosterone, the adrenal cortical salt-and-water-hormone par excellence, facilitates renal excretion of potassium while decreasing renal excretion of sodium (14). Hence the adrenal cortical activity during the lag in adjustment to the high sodium intake, though it prevented renal excretion of sodium, perhaps facilitated renal excretion of potassium. In subject E. W. the urine collection during the first 24 hours of sodium citrate administration was divided so that the urine passed during the first six hours of sodium citrate administration was collected separately and on analysis was found to contain only 1.4 mEq. of sodium (out of a total excretion of 215 mEq. for that 24 hour period) while it contained more than one-fourth of the total large potassium excretion of that day.

The negative intracellular sodium balance that followed the sodium chloride administration in subject E. W. is of sufficient magnitude to be interesting. The administration of large amounts of sodium chloride, which is "acid-producing" in that it contains equivalents of sodium and chloride (whereas the extracellular fluids normally contain sodium to chloride in the ratio of 1.4 to 1), apparently caused a transfer of intracellular so-

dium to the extracellular fluids which would serve to help combat this effect of sodium chloride administration. Such a mechanism assisting in the prevention of acidosis has also been described by Darrow (11, 15).

It is realized that the type of calculation made above would include any extrarenal or extrafecal sodium loss from the body as a gain in "intracellular sodium." This possibility reduces itself to losses of sodium through the skin. The studies of Freyberg and Grant (16) of the insensible losses of sodium from the skin of normal adult subjects show that the daily sodium loss is less than 10 mEq. Hence in our normal subjects who avoided exertion and sweating, a cumulative loss of sodium through the skin over the three-day periods of about 30 mEq. would scarcely affect the positive balances recorded.

IV. *Glomerular filtration, tubular reabsorption and the excretion of sodium*

There has been considerable discussion in the current literature regarding the possible roles played by change in glomerular filtration rate and tubular reabsorption of sodium in the formation of edema (17-19). Because our studies involved very low and very high rates of sodium and chloride excretion resulting from the marked variations in sodium intake, observations were made to determine, if possible, how glomerular filtration and tubular reabsorption of sodium affected the excretion of sodium by normal subjects. Table III shows the simultaneous 24 hour endogenous creatinine clearance taken as a measure of glomerular filtration rate (2, 3) and the simultaneous 24 hour urine sodium and chloride content. Simultaneous inulin clearances and sodium excretions for subjects M. L. and W. C. are also recorded.

It is obvious from a brief examination of these data that there is no correlation between glomerular filtration rate and renal excretion of sodium or chloride. Following the sodium chloride administration an increase in glomerular filtration rate is associated with an increased rate of excretion of sodium. However, no such increased rate is seen following the sodium citrate administration in spite of an equally large increase in the rate of sodium excretion. In another paper (1) we have suggested that the increased glomerular filtration rate

following sodium chloride administration is a non-specific response secondary to the resulting increased vascular volume. One certainly is not justified in interpreting it as a specific adjustment of the kidney for the purpose of increasing sodium excretion as has been postulated recently (18). Our findings in normal subjects are similar to those of Briggs *et al.* (19) who found no relation between glomerular filtration rates and sodium excretion in patients with congestive heart failure.

In what has just been said, mention of the serum sodium and chloride concentrations at the time of these clearance studies has been purposely avoided. Mokotoff *et al.* (18) have claimed that the tubular reabsorption of sodium is constant per unit of glomerular filtrate volume ("13.3 mM per 100 cc. of glomerular filtrate") and therefore varies directly with the glomerular filtration rate. These workers subtracted the sodium excreted in the urine from the product of the glomerular filtration rate and the serum sodium to obtain the rate of tubular reabsorption. As the quantity of sodium filtered is in nearly all cases tremendous in comparison with the quantity excreted such calculations are questionable. With a glomerular filtration rate of 160 liters in 24 hours and serum sodium of 140 mEq. per liter our subject E. W. was presenting approximately 22,400 mEq. of sodium to his renal tubules while the 24 hour urinary sodium excretion varied between 0.6 and 500 mEq. depending on the sodium intake. Thus as some 97% of the filtered sodium was always reabsorbed, the relatively large shift in urinary sodium must have occurred as a result of variations within this remaining 3% of the filtered sodium. In edematous cardiac and nephritic patients the glomerular filtration rate, though somewhat reduced (17-19), remains sufficiently large to still make the quantity of sodium excreted only a very small percentage of that filtered. Accordingly, the answer to the whole problem of the manner in which renal function is altered in certain cardiac and nephritic subjects making them unable to rid themselves of the usual daily sodium intake is hidden in the manner in which this very small fraction of the total filtered sodium is handled. However, methods such as the mannitol clearance with its unavoidable error of about 10% are much too crude to demonstrate these fine changes. One cannot

detect changes of 3% in a function by using a measuring stick with a 10% error.

CONCLUSIONS

Sodium as the chloride and as the citrate salts was administered to normal subjects maintained on very low sodium and chloride diets. The effects on body weight, sodium, potassium, chloride and nitrogen balances were recorded. Simultaneous glomerular filtration rates and urinary excretions of sodium and chloride were obtained.

The gain in body weight with sodium chloride was strikingly greater than with sodium citrate. Ingestion of sodium as chloride caused parallel increases in urinary excretion of sodium and chloride, whereas sodium as citrate resulted in a similar increase in urinary sodium but the urinary chloride did not increase. Since in the previous paper (1) it was concluded that the desoxycorticosterone-like hormone activity of the adrenal cortex is depressed by sodium administration, this dissociation of sodium and chloride excretion demonstrates that some other factor causes renal conservation of chloride.

When sodium was administered as the citrate salt, a large increase in body sodium occurred accompanied by a large diuresis of potassium. Appropriate calculations indicate that significant amounts of sodium entered the cells, displacing large amounts of potassium.

The quantities of sodium appearing in the urine from 0.6 to 4.77 mEq. bore no relationship to the simultaneous volumes of glomerular filtrate. The sodium present in the urine is 3% or less of the sodium delivered to the tubules in the glomerular filtrate. Accordingly the relatively small variation in the amount of sodium not reabsorbed determines whether body sodium will be increased or decreased. Since the error inherent in the present methods is greater than the function to be measured, the exact role of filtration rate and tubular reabsorption in the excretion of sodium cannot at the present time be defined.

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FURTHER STUDIES WITH THE SALT TOLERANCE TEST IN NORMAL INDIVIDUALS AND IN PATIENTS WITH ADRENAL CORTICAL HYPERFUNCTION

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In a previous paper (1) we reported on the utilization of intravenously injected salt before and after the injection of desoxycorticosterone acetate in normal individuals and in patients with Cushing's syndrome. It was pointed out that the injection of this steroid in normal individuals resulted in a decrease in the urinary excretion of sodium and chloride ions as compared to the control values. This is the expected and orthodox effect. On the other hand, the injection of this compound in patients with Cushing's syndrome under the circumstances of our test resulted in an increase in the urinary excretion of sodium and chloride.

Since the publication of the original paper several reports have appeared describing a similar phenomenon occurring under a variety of conditions. Reifenstein (2) has noted a paradoxical salt loss in patients with Cushing's syndrome treated with testosterone. Kriss and Futcher (3) have observed that in two of three patients with Cushing's syndrome, the rate of urinary excretion of sodium, chloride, and water following the rapid intravenous administration of 400 c.c. of 5% saline exceeded that observed in similarly treated control subjects. In the patients with Cushing's syndrome the increased excretion of the ions and water was accompanied by a reduction in renal tubular reabsorption of these substances, as compared to the controls. In addition, these authors report the instances of two obese females with arterial hypertension in whom the salt tolerance test, employing our technique, was positive at the time of menstruation and negative in the same patients during the intermenstrual period. They suggest that during the menstrual cycle there is a temporary preponderance of adrenocortical influence. In one patient with Cushing's syndrome the salt tolerance test resulted in a normal salt retaining response, despite the fact

that the patient exhibited salt diuresis during the clearance study performed five days previously. More recently, Forsham and his coworkers (4) confirmed the paradoxical salt loss following the injection of desoxycorticosterone acetate in patients with Cushing's syndrome. They suggested that the mechanism of this phenomenon was perhaps dependent upon a competitive renal inhibition of desoxycorticosterone as against some other non-salt retaining adrenal cortical fractions.

The mechanism of this paradoxical effect is obscure. We have speculated that it is at least theoretically possible that in some patients with adrenal cortical hyperfunction excessive amounts of salt-retaining fractions, manufactured by the increased activity of the adrenal cortex or injected from an exogenous source, are converted into substances lacking salt-retaining effects. The basic structural similarity between those adrenal cortical steroids exercising a salt retaining effect and those lacking it would suggest that the conversion of one into another is perhaps chemically feasible.

METHOD

The method of study employed is that described previously (1). "All tests were conducted according to the following standard routine: The patients were permitted no food throughout the period of observation and no fluid after 7 P.M. the night before. At 6 A.M. on the morning of the studies the patient was asked to void and the urine was discarded. He was then given 500 c.c. of water to drink at one time, and all of the urine voided was collected over a three-hour period to 9 A.M., carefully labeled and set aside. At 9 A.M., 200 c.c. of 5% saline (10 grams of salt) was injected intravenously and the urine voided during the next three hours, to 12 M., was similarly collected and labeled. Two days later the same procedure was repeated, but, in addition, the patient received 10 mgm. of desoxycorticosterone acetate intramuscularly the previous evening at 10 P.M. The urine volume of each period was carefully measured and sodium and chloride determinations were made on each specimen. Sodium was determined according to the method

of Butler and Tuthill (5), and chlorides according to the method of Harvey (6). The total urinary sodium and chloride ions excreted during the 6 A.M. to 9 A.M. period were subtracted from those excreted between 9 A.M. and 12 M. (period after the intravenous injection). The difference represents the excess over the basal excretion of these ions. This figure in milliequivalents, divided by 171 (*i.e.*, milliequivalents in 10 grams of sodium chloride) yields the fraction of the injected ions excreted (Na_e and Cl_e). The comparison of the results obtained during the control period and that after the injection of desoxycorticosterone acetate shows the percentage of increased retention resulting from the use of the hormone."

Twenty-two normal controls and ten patients with Cushing's syndrome were studied in the present series. The diagnosis of Cushing's syndrome in every instance of this group was established by the presence of a classical clinical picture plus the demonstration, by perirenal insufflation or surgical exploration or both, of an adrenal tumor or bilateral adrenal enlargement. Patients with Cushing's syndrome need manifest no overt gross or histological adrenal cortical abnormalities. For purposes of clarity and definition, however, we have limited our present study to those patients who manifested the usual typical clinical picture and anatomical evidence of adrenal disease. Finally, there are probably many abortive forms of either transient or permanent adrenal cortical hyper-

TABLE I

Subjects	Control				After injection of 10 mgm. of desoxycorticosterone acetate				Excretion				Serum sodium m.eq/l
	Urine volume		Injected Na excreted Na _e	Injected Cl excreted Cl _e	Urine volume		Injected Na excreted Na _d	Injected Cl excreted Cl _d	Decrease		Increase		
	6 to 9 A. M.	9 to 12 A. M.			6 to 9 A. M.	9 to 12 A. M.			$100 \frac{(\text{Na}_e - \text{Na}_d)}{\text{Na}_e}$	$100 \frac{(\text{Cl}_e - \text{Cl}_d)}{\text{Cl}_e}$	$100 \frac{(\text{Na}_d - \text{Na}_e)}{\text{Na}_e}$	$100 \frac{(\text{Cl}_d - \text{Cl}_e)}{\text{Cl}_e}$	
	c. c.	c. c.			%	%			c. c.	c. c.	%	%	
<i>Normals</i>													
I. M.	395	232	10.9	11.8	405	182	6.7	8.0	38.5	32.5			
W. K.	260	950	19.1	18.4	245	770	11.8	13.5	38.3	26.5			
M. W.	58	128	8.9	9.5	78	38	0.4	0.4	95	110			
J. M.	755	170	6.7	7.4	560	283	8.6	5.4		27	28.5		
R. B.	133	168	5.1	6.2	140	220	6.4	5.3		14	13.8		
W. C.	485	360	12.1	12.2	495	40	-14.5	-14.5	220	220			
B. A.	90	102	1.5	2.9	72	132	6.3	9.3			188	220	
A. R.	420	415	19.1	15.6	558	352	15.7	16.7	17.8			7.3	
V. V.	430	450	19.4	23.9	395	140	0.9	0.5	95	98			
F. K.	330	375	30.5	34.2	260	260	27.2	31.7	10.8	10.2			
C. L.	260	370	20.4	24.0	340	180	1.5	0.7	93	97			
F. B.	180	100	1.2	2.3	253	134	1.1	2.3	8.4	0			
T. D.	390	472	39.1	42.9	32	84	6.0	8.4	85	76			
S. W.	508	352	11.7	14.6	720	218	-0.1	-3.9	105	126			
H. L.	378	560	34.8	30.8	562	218	9.6	12.8	73	58			
I. P.	110	170	8.2	10.2	55	105	6.5	8.6	20.7	15.4			
E. S.	124	235	17.3	17.7	230	183	7.3	4.7	59	74			
P. K.	105	170	12.6	12.8	120	105	6.4	4.5	49	64			
A. D.	650	290	21.5	28.7	465	170	11.9	16.7	43.5	42			
N. W.	300	155	5.6	5.6	200	118	1.1	0.1	80	98			
A. L.	200	187	15.6	18.5	250	44	-0.7	-1.8	104	110			
P. P.	698	323	24.3	16.7	940	410	8.8	9.1	66.5	45.5			
<i>Cushing's syndrome</i>													
R. S. (1)	490	560	19.8	20.5	590	585	19.0	16.7	4.1	18.6			148.4
R. S. (2)	355	650	33.3	30.8	475	535	16.0	15.6	52	49.5			151
R. K.	840	480	24.5	28.1	1060	620	26.3	27.3		4.4	7.3		158.2
D. F.	530	400	4.4	5.3	305	190	1.8	2.7	59	49.5			156.3
H. P.	510	295	22.0	19.5	644	400	41.6	43.4			89	122	141.5
A. L.	385	365	38.0	39.5	375	410	44.7	44.8			17.6	13.4	141
F. P.	390	307	14.6	19.1	277	392	28.6	34.5			96	82	137.1
T. S.	255	190	10.8	14.8	274	365	39.6	36.1			280.7	143.8	140.1
A. B.	370	380	14.6	10.1	685	310	-1.9	-0.5	113	105			141.3
S. G.	600	350	8.2	8.7	470	1000	44.9	43.1			448	407	142.2
J. S.	342	130	5.9	5.9	315	124	7.8	9.1			32.2	54.2	
A. P.	378	336	8.9	9.1	380	418	19.8	19.5			122.4	114.2	
E. S.	245	190	26.7	32.0	297	950	70.3	59.8			163.2	87	
E. G.	650	250	11.8	12.2	418	238	17.3	19.0			46.6	55.7	
J. R.	168	534	37.7	35.4	115	745	55.3	56.4			74	60	
<i>After radiotherapy</i>													
J. R.	485	745	63.3	49.5	340	870	60.9	56.5	4.1			14.2	
S. S.	244	300	20.2	28.0	257	95	-2.1	-4.6	110	116			139.8

function in which the clinical picture is ill defined, more subtle, and less complete. The response of such patients to the salt tolerance test will await further study.

RESULTS

In the table are presented the results obtained in the normal individuals and in the 15 patients with Cushing's syndrome. Five of these latter patients were discussed in our previous report (1). In one of the present control series there occurred a diuresis of chloride and sodium following the administration of desoxycorticosterone acetate. In three others there was a minimal diuresis of one or the other of these two electrolytes, but a retention of the other electrolyte.

In ten of the 15 patients with Cushing's syndrome there occurred a diuresis of sodium and chloride following the administration of desoxycorticosterone acetate under the conditions of our test. Five patients failed to show the characteristic response. Of this group of five individuals, one showed a minimal diuresis of sodium but a considerable retention of chloride. Two of the remaining four patients had received intensive X-ray therapy to the hypophysis prior to the performance of the test. In one of these two patients the salt tolerance test was performed both before and after X-ray therapy to the pituitary. Before irradiation there occurred a marked diuresis of sodium and chloride following the administration of the compound, while the repetition of the test after conclusion of X-ray therapy resulted in a retention of sodium and a reduction in the chloride diuresis.

Serum sodium determinations were made in nine of the patients with Cushing's syndrome. Three of this group showed a considerable elevation of the serum sodium, while in the remaining six instances the blood electrolyte values were well within the normal range. One of the latter group and all three of the former showed a retention of sodium or chloride, or both, following the injection of the desoxycorticosterone acetate.

SUMMARY

1. The salt tolerance test was performed on 22 normal individuals and on 15 patients with adrenal cortical hyperfunction.
2. In one of the control series there occurred

a diuresis of sodium and chloride following the administration of desoxycorticosterone acetate under the conditions of our test. In three others there was a minimal diuresis of one or the other of these two electrolytes, but a retention of the other electrolyte.

3. In ten of the 15 patients with adrenal cortical hyperfunction there occurred a diuresis of sodium and chloride following the administration of the hormone.

4. Two of the five patients who failed to show the characteristic response had received intensive X-ray therapy to the pituitary prior to the performance of the test. In one of these patients the test was performed both before and after X-ray treatment. Prior to X-ray therapy the administration of desoxycorticosterone acetate resulted in a diuresis of sodium and chloride, while after treatment there occurred a retention of sodium and a reduction in the degree of chloride diuresis.

5. Blood sodium determinations were made in nine patients with adrenal cortical hyperfunction. In three there occurred a considerable elevation of this electrolyte, while in the remaining six instances the serum sodium was well within the normal range.

6. All three of the former group and one of the latter showed a retention of sodium or chloride or both.

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THE ACTION OF STREPTOCOCCAL DESOXYRIBOSE NUCLEASE (STREPTODORNASE),¹ *IN VITRO* AND ON PURULENT PLEURAL EXUDATIONS OF PATIENTS²

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In previous reports it has been demonstrated that desoxyribose nucleoprotein is a significant constituent of the solid sediment of purulent exudates (1), and that hemolytic streptococci elaborate, during their growth in broth cultures, a desoxyribose nuclease (streptodornase) in considerable quantities (2). The latter finding was also described independently and concomitantly by McCarty (3).

In a more recent report (4) results have been described which were obtained when partially purified streptococcal concentrates, containing both streptokinase and streptodornase were injected into the pleural cavity of patients suffering from various types of diseases causing fibrinous, purulent, and sanguinous exudations.

The concentrates employed³ were prepared according to methods developed and described by Christensen (5). Since they contained both the fibrinolytic principle, streptokinase, and the pus-liquefying enzyme, streptodornase, it was possible to demonstrate that two enzymatic systems were operative when introduced locally within the areas of disease. Fibrin and fibrinogen were predominant in cases of uninfected loculated hemothorax; desoxyribose nucleoprotein was most conspicuous in cases with thick coarse purulent exudates; in other cases both substrates were present in varying amounts depending on the nature of the underlying disease.

With respect to the streptodornase activity, the earlier reports (1, 4) described the depolymerization of desoxyribose nucleoprotein and desoxyribose nucleic acid, and also demonstrated de-

creases in the amount of the solid sediment of purulent exudates in association with marked drops in the viscosity of the same specimens following intrapleural injections of the streptococcal concentrates.

It is the purpose of this article to describe in detail the biochemical changes induced by the action of streptodornase in association with the characteristic physical changes that have attended depolymerization, and, in addition, the histological and cytological changes that have been made evident by the use of microscopic preparations serially stained by the Feulgen method (6).

The data to be presented were derived from:

1. Determinations of the action of streptodornase on purified desoxyribose nucleic acid derived from calf thymus.
2. Observations of the action on purulent exudates produced, *in vivo*, following the intrapleural injection of streptococcal concentrates containing streptodornase; the effects, *in vivo*, have also been paralleled by comparable experiments conducted *in vitro*, by incubating streptodornase with specimens of purulent exudation derived from patients.

MATERIALS AND METHODS

Streptodornase. This enzyme was contained in the partially purified streptococcal concentrates that have been used throughout these studies. The concentrates also contained streptokinase in measurable quantities. However, the effects to be described in this article are limited to those caused by the nuclease. A unit of streptodornase has been arbitrarily defined as the amount that will cause a drop of one viscosity unit in 10 minutes at 37° C in an enzyme substrate mixture consisting of 2.5 cc. of desoxyribose nucleic acid and 0.1 cc. enzyme solution. The substrate consisted of 0.15–0.2% solution of thymus desoxyribose nucleic acid in M/40 barbital buffer and 0.003 M Mg⁺⁺ at pH 7.4. The substrate had an initial viscosity relative to water of 3–5 units.

The amounts of the enzyme employed will be given in individual protocols.

¹ The word "Streptodornase" has been employed in this article as an abbreviation of streptococcal desoxyribose nuclease.

² This study was supported by a grant from the National Institutes of Health, United States Public Health Service.

³ A considerable amount of the preparations was supplied by Lederle and Co.

Desoxyribose nucleic acid. This was prepared from calf thymus by the method of Mirsky and Pollister (7). A 0.15–0.20% solution in M/40 barbital buffer was heated at 56° C for two hours, to destroy any residual depolymerase activity, before its use as a substrate.

Viscosity. Viscosity measurements were done in a constant temperature bath at $37 \pm 0.2^\circ$ C. For purified desoxyribose nucleic acid solutions or thin exudates, Ostwald viscosimeters with a flow time of 10–15 seconds at 37° C were employed. For thick exudates an LV Model Brookfield viscometer was employed. Results are expressed in units of viscosity $\times \text{H}_2\text{O}$.

pH. pH was determined by glass electrode using a Beckmann pH meter.

Sediment. The per cent sediment was determined by filling Wintrobe tubes with exudate and spinning at 2500 RPM for one hour.

Phosphorus. Total acid soluble phosphorus was determined by a modification of the method of Fiske and SubbaRow (8) on digested 8% trichloroacetic acid filtrates. Inorganic phosphorus was determined by a modification of the method of Fiske and SubbaRow (8) on 8% trichloroacetic acid filtrates. Organic acid soluble phosphorus was obtained by subtracting the inorganic P from the total acid soluble P.

Acid soluble nitrogen. Determined on 8% trichloroacetic acid filtrates by micro Kjeldahl and nesslerization.

Ammonia. Determined by method of Van Slyke and Cullen (9).

Uric acid. Determined on 1% tungstic acid filtrates by the method of Folin (10).

Absorption at 2600 Å. Determined on trichloroacetic acid filtrates in a Beckmann Spectrophotometer.

Feulgen stain. An adaptation of Rafalko (11) and Stowell's (12) modification of the method originally described by Feulgen (6) has been regularly employed.

Solutions

1. *Leuco-fuchsin reagent.* 0.5 Gm. basic fuchsin suspended in 100 cc. boiling distilled water. Shake. Cool to 50° C. Filter. Decolorize by slowly bubbling SO_2 through the solution for one to two hours. Store in dark for 24 hours. Add 0.25 Gm. activated charcoal (preferably norit). Shake for one minute and filter rapidly through coarse filter paper. Store in icebox in dark bottle.
2. *1N HCl solution.*
3. *Sulfurous acid solution.* Bubble SO_2 through 1 liter of distilled water for three to four hours in the cold. Store in icebox in dark bottle.
4. *Fast Green F.C.F. solution*—0.02% alcoholic solution.

Technique⁴

1. Thick and thin smears of exudate are made and air dried.

⁴ All procedures are carried out at room temperature unless otherwise specified.

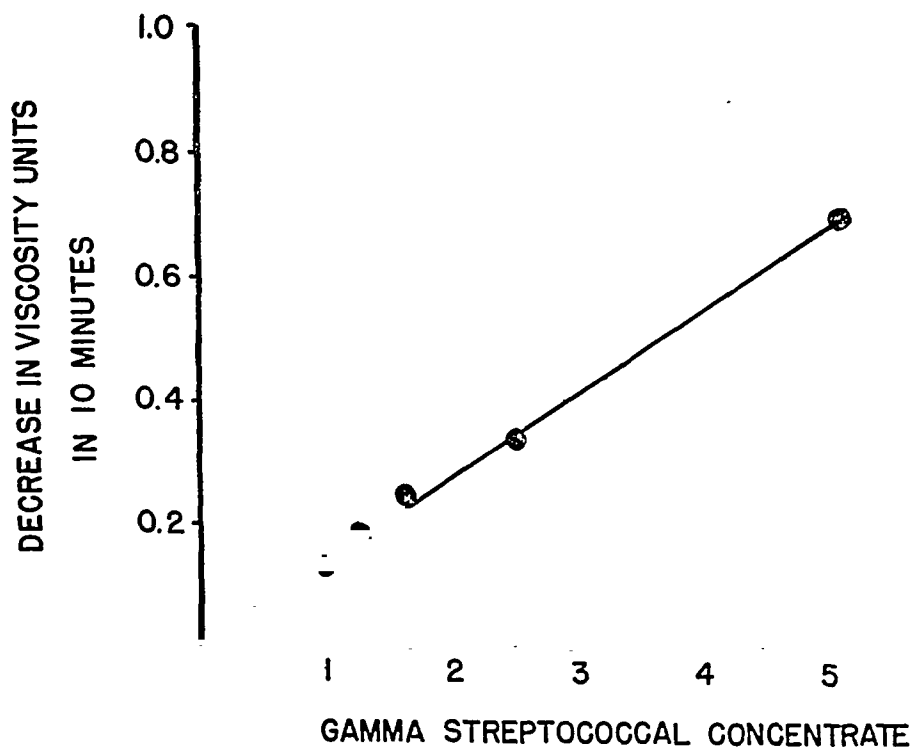


FIG. 1. THE EFFECT OF VARYING AMOUNTS OF STREPTODORNASE CONCENTRATE ON THE DEPOLYMERIZATION OF PURIFIED DESOXYRIBOSE NUCLEIC ACID

2. Immerse in 1N HCl for one minute.
3. Immerse in 1N HCl for 15 minutes at 56° C.
4. Rinse in distilled water.
5. Rinse in sulfurous acid solution.
6. Immerse in leuco-fuchsin solution for two hours.
7. Rinse in three successive baths of sulfurous acid solution for one minute each.
8. Immerse in tap water for five minutes and then rinse with distilled water.
9. Immerse in Fast Green solution for 20-30 seconds.
10. Rinse in distilled water, and air dry.

RESULTS

1. The action of streptodornase on purified desoxyribose nucleic acid (calf thymus).

In Figure 1 the depolymerase activity of 1 to 5 gamma of a streptodornase concentrate in 0.1 cc. was tested with 2.5 cc. of a 0.15% solution of desoxyribose nucleic acid in M/40 barbital buffer containing 0.003 M of Mg^{++} at pH 7.4. The tests were conducted for periods of ten minutes in a water bath at 37°C. From the data it may be noted that a linear relationship between concentration of enzyme and depolymerase activity was demonstrable.

In Figure 2 the effect of temperature on the depolymerase activity of the streptococcal concentrates is illustrated.

For the experiment on thermal effects, 0.1 cc. of a 1:1000 dilution of a 5% crude streptococcal concentrate, and containing approximately 1.4

units of streptodornase was added to 2.5 cc. of a 0.2% solution of desoxyribose nucleic acid in M/40 barbital buffer (pH 7.4) containing 0.003 M of Mg^{++} . Measurements of viscosity were made over periods of ten minutes following mixture of enzyme and substrate.

As may be noted from Figure 2 the rate of depolymerization progressively increased with rising changes in temperature reaching a maximum at 45° C. Above 45° C the rate of enzyme inactivation exceeded the increased velocity of reaction. The activity of the enzyme rapidly fell off as the thermal death point of 56° C was approached. Temperature inactivation of the enzyme appeared to begin above 30° C.

In Figure 3 the relation of pH to streptodornase activity is demonstrated.

To a 0.2% solution of desoxyribose nucleic acid in M/40 sodium barbital containing 0.003 M of Mg^{++} , 3% acetic acid was added in different amounts in order to obtain solutions of substrate varying in pH from 5.2 to 9.0. 0.1 cc. of a 1:1000 dilution of a 5% preparation of concentrate and containing 1.8 units of streptodornase was added. Measurements of viscosity were made over periods of ten minutes in a water bath at 37° C following the mixture of enzyme and substrate. The final pH was checked before and at the end of each experiment.

As may be noted from the data in Figure 3, activity was greatest between pH 7.0 and 8.5.

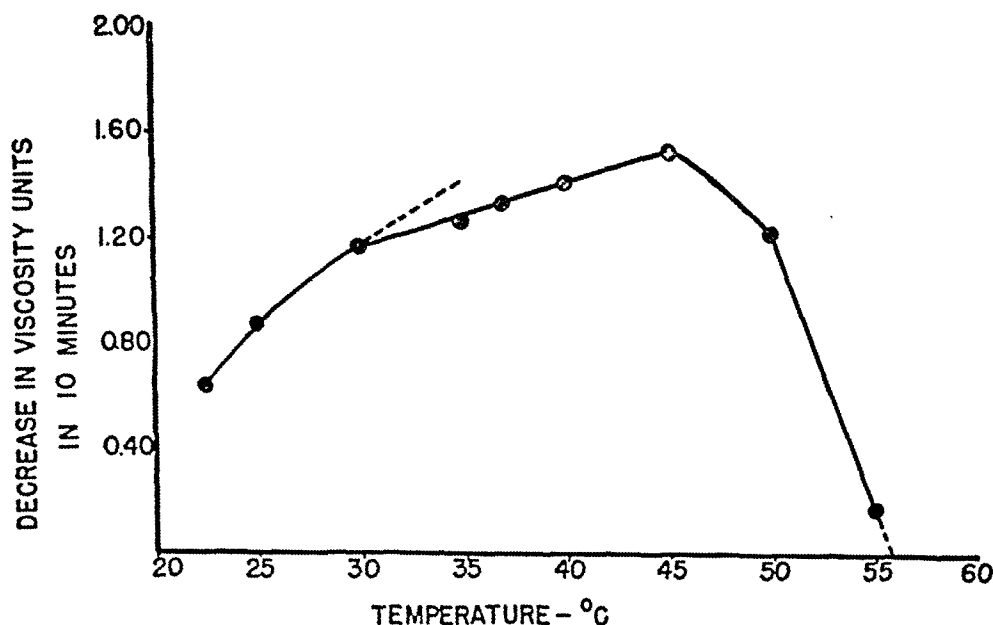


FIG. 2. THE EFFECT OF TEMPERATURE ON THE DEPOLYMERASE ACTIVITY OF STREPTODORNASE

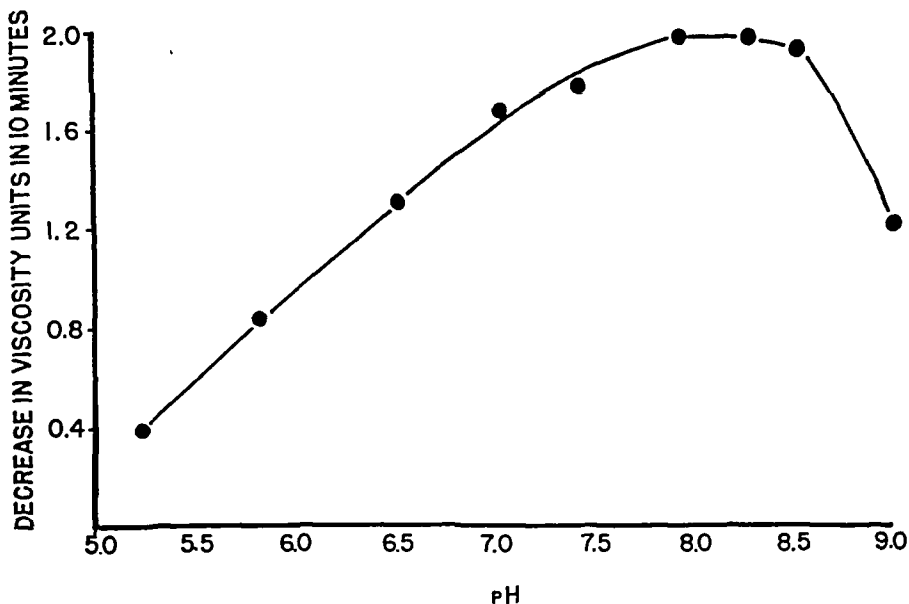


FIG. 3. THE EFFECT OF pH ON THE DEPOLYMERIZATION OF PURIFIED DESOXY-RIBOSE NUCLEIC ACID BY STREPTODORNASE

In Figure 4, the results of an experiment are summarized that was conducted for the purpose of determining whether acid soluble phosphorus and acid soluble nitrogen were liberated by the action of streptodornase.

0.2 cc. of a streptococcal concentrate containing 180 units of streptodornase per cc. was incubated at 37° C with 67 cc. of a 0.2% solution of purified desoxyribose nucleic acid (calf thymus) containing 0.003 M of Mg^{++} in M/40 barbital buffer at pH 7.4.

From the findings recorded in Figure 4 it may be seen that analyses of trichloroacetic acid filtrates revealed a progressive liberation of organic acid soluble P and acid soluble N and that the activity continued after depolymerization, as determined by viscosity measurements, was completed. Since no significant increase in inorganic P was noted, the rise in total acid soluble P may be interpreted as being entirely dependent on an increase in phosphate esters.

No evidence of deamination was observed. These findings, coupled with the observation of marked increases in the extinction coefficient at 2600 Å of trichloroacetic acid filtrates of desoxyribose nucleic acid incubated with streptodornase, indicate a liberation of acid soluble nucleotides.

A search for inhibitors has been made using a wide variety of reagents that might be pertinent to

the broad problems of this study including the possible significance of inactivation in relation to the effective treatment of patients.

The following substances were found to have no effect on the depolymerase activity of streptodornase: salicylate, gentisate, ascorbate, thiourea, paraminobenzoic acid, parachlorophenol, oxalate, colchicine, and penicillin. In conducting the above experiments 0.1 cc. solution of the test reagents was mixed with the nucleic acid substrate. 0.1 cc. of streptodornase solution containing 1 unit of enzyme was then added and the rate of depolymerization over a ten minute interval observed. The quantities of each reagent employed were estimated as being of sufficient concentration to give an indication of an inhibitory effect if such were present. Since no evidence of inhibition has been found, the observations have not been extended beyond the preliminary stages.

Definite evidence of an inhibitory effect has, however, been noted in the use of citrate and heparin.

The results briefly stated are shown in Table I.

2. The action of streptodornase on the purulent exudates of patients, *in vivo* and *in vitro*

Nine patients with empyema have been studied. The diseases giving rise to the empyema were caused by tuberculosis in four instances, anaerobic

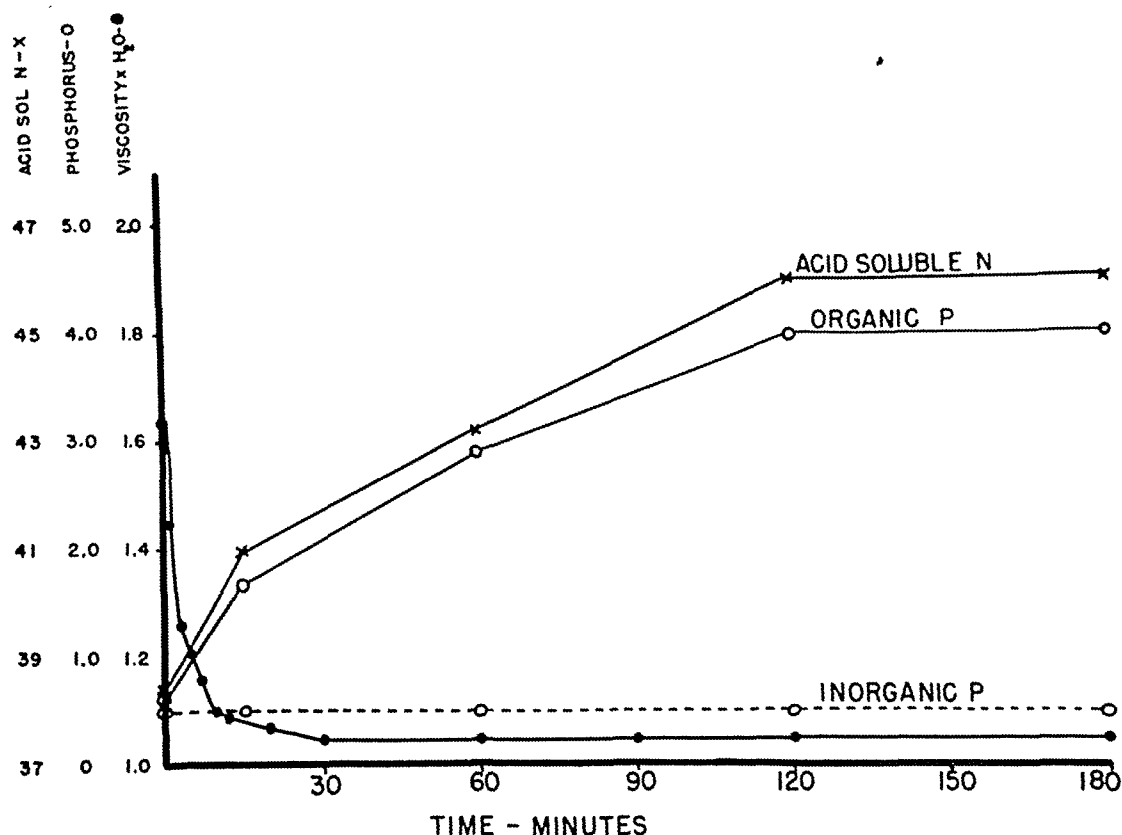


FIG. 4. THE EFFECT OF STREPTODORNASE ON THE DEPOLYMERIZATION AND LIBERATION OF ACID SOLUBLE NITROGEN AND PHOSPHORUS FROM PURIFIED DESOXYRIBOSE NUCLEIC ACID

streptococcus in two cases, pneumococcus pneumonia with sterile empyema in two cases, and infection with *B. pyocyaneus* in one case.

In the study of each case a sample of pleural exudate was removed for analyses prior to the injection. The streptococcal concentrates in amounts of fluid varying from 2 to 10 cc. and containing from 5000 to 50,000 units of streptodornase were introduced into the pleural site of the infection. Subsequent specimens were removed by

thoracentesis during periods of one or more hours in the first day and then usually at daily intervals thereafter for the duration of the observations.

The required dosage of streptodornase in relation to the type and amount of pus present in each case has not yet been determined on an exact quantitative basis. However, the doses employed, usually averaging 20,000 units per dose, have regularly proved effective as evidenced by the definitive changes to be described.

It may be noted at this point that no serious untoward effects occurred in any of the patients. The reactions were limited to pyrogenic ones of a transient nature together with febrile malaise such as that previously described (4). A report of the therapeutic effects of the injections together with the clinical details of the course of the patients is now in preparation.

In six cases of empyema measurements were made of the liberation of organic acid soluble P at the site of the pleural exudation following the local introduction of the streptococcal concentrates containing streptodornase.

Determinations of acid soluble P and N were made on the supernatant portion of centrifuged

TABLE I
Inhibition of streptodornase depolymerase activity by various reagents

Substance tested	Amount in test mgms.	Inhibition %
Salicylate	0.02	0
	0.2	10
	2.0	0
Gentisate	0.1	1
	0.2	5
	1.0	0
Thiourea	1.0	1
Oxalate	1.0	0
Colchicine	0.011	0
Paraaminobenzoate	1.0	0
Para-chlorophenol	0.25	8
Ascorbate	1.0	16
Penicillin	(10,000 units)	19
Heparin	1.0	61
Citrate	1.0	82

samples of exudate removed at hourly intervals. From the data in Figure 5 it may be seen that the peak of the rise in liberated P was reached one to four hours after the injection. The values then returned to the pre-injection level during the next 24 hours.

The liberation of acid soluble N was also evident coincidental with the rise in P. The acid filtrates of the supernatant specimens showed marked increases in absorption at 2600 Å. Further breakdown to inorganic P or uric acid was not demonstrable, as shown in Figure 6.

Experiments comparable to those just described have been conducted, *in vitro*, by incubating mixtures of streptodornase and pus, and making determinations of acid soluble P and N liberated into the supernatant fluid. The results of such a study

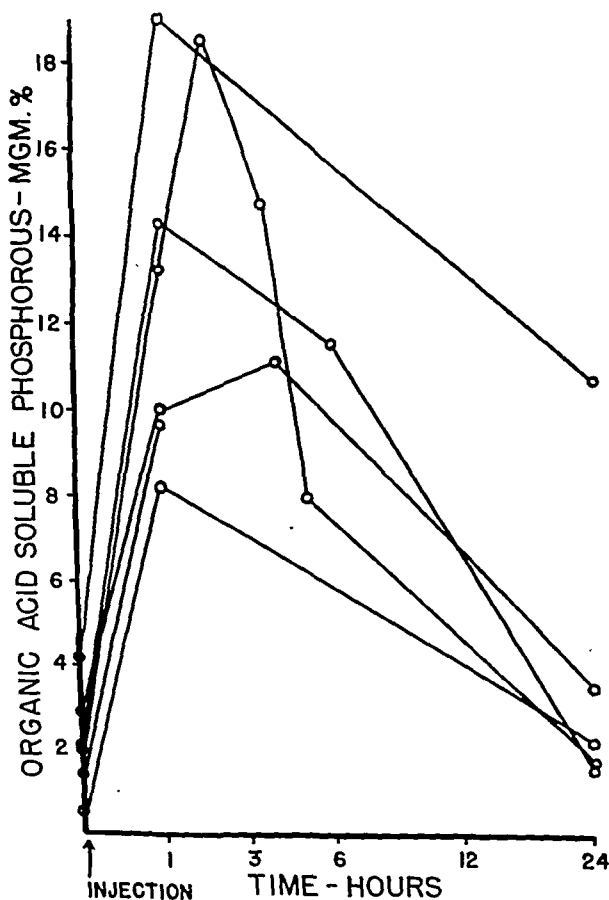


FIG. 5. THE LIBERATION OF ORGANIC ACID SOLUBLE PHOSPHORUS INTO THE SUPERNATANT OF PLEURAL EXUDATES AT VARYING INTERVALS FOLLOWING THE LOCAL INTRODUCTION OF STREPTODORNASE INTO THE SITE OF DISEASE IN SIX PATIENTS WITH EMPYEMA

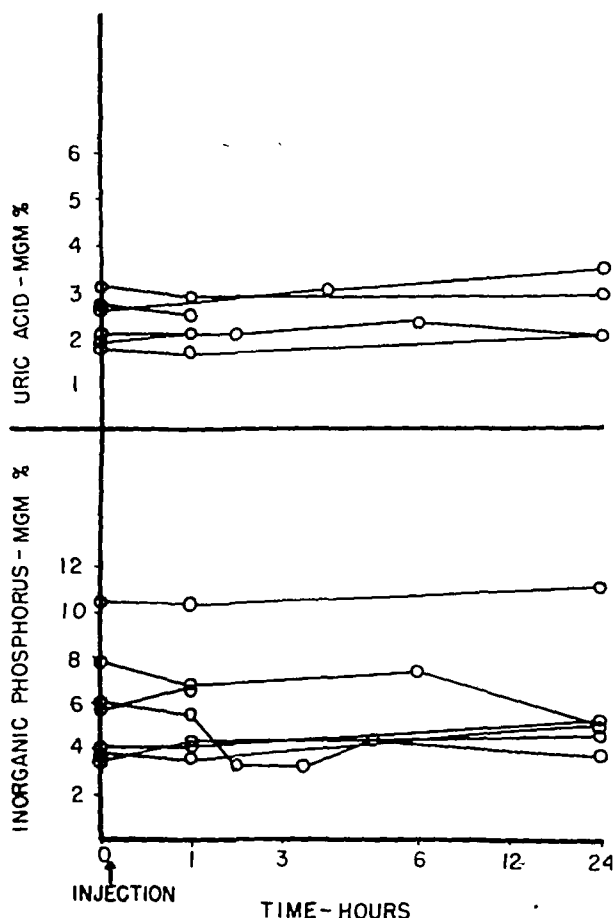


FIG. 6. THE CONCENTRATION OF URIC ACID AND INORGANIC PHOSPHORUS OF THE SUPERNATANT OF PLEURAL EXUDATES AT VARYING INTERVALS FOLLOWING THE LOCAL INTRODUCTION OF STREPTODORNASE INTO THE SITE OF DISEASE IN PATIENTS WITH EMPYEMA

are recorded in Table II. The purulent exudate was obtained from a case of penicillin treated pneumococcus Type II pneumonia who developed a sterile empyema. The pH of the fluid was 6.5. The sample of pus was divided into three aliquots. One aliquot served as control. A second aliquot was incubated with a streptococcal concentrate previously heated at 56° C for one and one-half hours to destroy the desoxyribonuclease present. This concentrate still contained considerable streptokinase. The third aliquot was incubated with the unheated streptococcal concentrate containing both streptokinase and desoxyribonuclease.

The control exudate, and the exudate mixed with concentrate free of streptodornase, liberated acid soluble N and P into the supernatant at about the same rate. The concentrate containing strepto-

TABLE II

The in vitro effect of streptodornase on the liberation of acid soluble P and N into the supernatant of a purulent exudate

Experimental	Streptodornase added	Streptokinase added	Time of incubation	Acid soluble N		Total acid soluble P		Inorganic P		Organic acid soluble P	
				Total	Change	Total	Change	Total	Change	Total	Change
	units	units	min.	mgm. %	mgm. %	mgm. %	mgm. %	mgm. %	mgm. %	mgm. %	mgm. %
49 cc. pus + 1 cc. M/40 barbitol buffer	0	0	0	57.5	—	13.0	—	9.6	—	3.4	—
			180	75.0	17.5	14.9	1.9	11.0	1.4	3.9	0.5
49 cc. pus + 1 cc. heated streptococcal concentrate in M/40 barbitol buffer	0	10,000	15	61.6	4.1	13.1	0.1	9.9	0.3	3.3	0.0
			30	61.6	4.1	13.8	0.8	9.8	0.2	4.0	0.6
			60	64.0	6.5	13.9	0.9	10.0	0.4	3.9	0.5
			120	68.6	13.1	13.9	0.9	10.4	0.8	3.5	0.1
			180	68.6	13.1	13.8	0.8	10.5	0.9	3.3	0.0
49 cc. pus + 1 cc. streptococcal concentrate in M/40 barbitol buffer	10,000	50,000	15	73.0	15.5	21.2	8.2	9.5	0.0	11.7	8.3
			30	75.0	17.5	23.3	10.3	9.5	0.0	13.8	10.4
			60	79.6	22.1	25.6	12.6	9.7	0.1	15.9	12.5
			120	95.6	38.1	28.2	15.2	10.2	0.6	18.0	14.6
			180	106.0	48.5	29.2	16.2	10.5	0.9	18.7	15.3

dornase produced a striking progressive liberation of organic acid soluble P and acid soluble N into the supernatant. The liberation of inorganic P was at the same rate in all three instances.

The above data point to the fact that since no liberation of inorganic P or uric acid was detectable in the supernatant portions of the exudates,

either *in vivo* or *in vitro*, the breakdown of the nucleoprotein of the exudates probably did not proceed further than the nucleotide stage.

A readily obvious physical change caused by the action of streptodornase on the intrapleural purulent exudates is the transformation of the viscid, thick, coarsely granular material to a thin, milky type of fluid. Photographs contained in previous articles (2, 4) demonstrate the extent of the visible change.

Quantitative estimations of the physical changes have been made by determining in the serial samples of exudate derived from the patients the decreases in both the per cent of sediment and viscosity in association with the biochemical changes described above.

Figure 7 demonstrates the effect of the injection on the viscosity of exudates in nine patients.

The range of fall was from 2–2400 viscosity units. Where the initial viscosities were highest because of large amounts of extracellular desoxyribose nucleoprotein, the effect was most striking. This correlation will be demonstrated in the subsequent description of the findings obtained by the use of Feulgen stained preparation.

The range of the per cent fall in one hour in the amount of sediment following injection in nine patients is shown in Figure 8. The observations were made by centrifuging aliquot parts of the serial samples of exudates at approximately 2500 revolutions per minute for 60 minutes.

The results are again closely correlated with

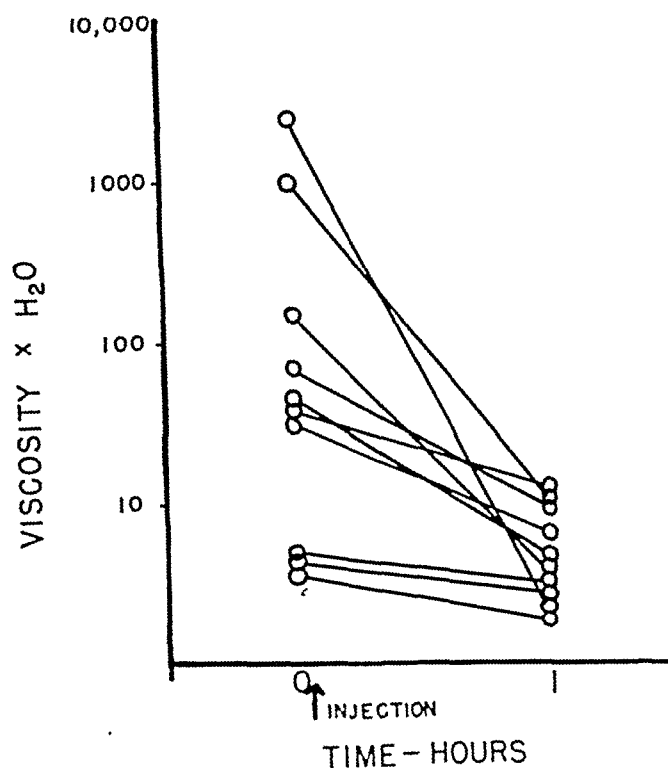


FIG. 7. THE EFFECT OF STREPTODORNASE, INTRAPLEURALLY INJECTED, ON THE VISCOSITY OF THE PLEURAL EXUDATES IN NINE CASES OF EMPYEMA

One patient was injected twice.

both the changes in viscosity and the microscopic demonstration of the lysis of extracellular desoxyribose nucleoprotein.

By the Feulgen method of staining, the presence of desoxyribose nucleoprotein has been identified microscopically as being present extracellularly in considerable abundance. Morphologically it appears, as has been previously described (1), in the form of small granules, fibrous-like strands, and amorphous plaques. Intracellularly, desoxyribose nucleoprotein is noted as a significant part of the nuclei. The extracellular material is obviously derived from destroyed cells, chiefly from the leucocytes of the exudate and to an undetermined degree from local fixed tissue cells. From the cytological studies, leucocytes in various stages of degeneration have also been noted.

The small granular forms of the desoxyribose nucleoprotein have been found to be characteristic of the freshest exudates, the reticular and amorphous forms to be present in greatest abundance in protracted cases of purulent infection such as tuberculous empyema.

When the streptococcal concentrates containing streptodornase have been introduced intrapleurally

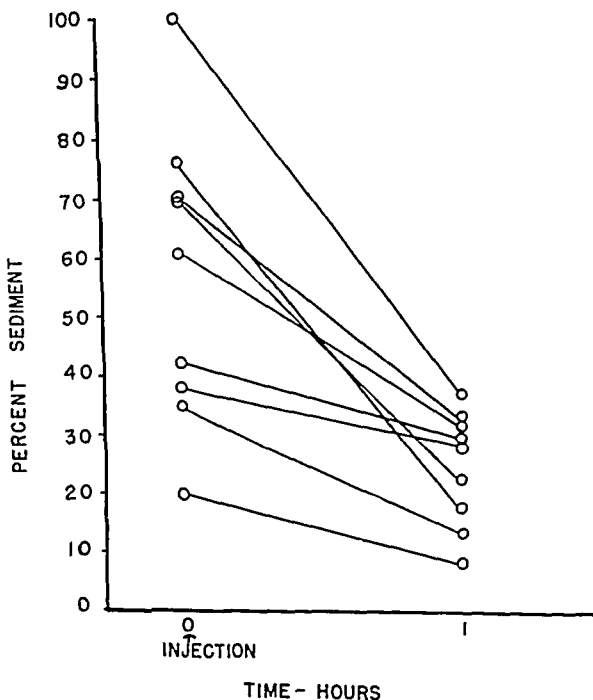


FIG. 8. THE EFFECT OF STREPTODORNASE, INTRAPLEURALLY INJECTED, ON THE PERCENTAGE SEDIMENT OF PLEURAL EXUDATES IN NINE CASES OF EMPYEMA



FIG. 9. FEULGEN STAIN OF PLEURAL EXUDATE ASPIRATED FROM A PATIENT WITH A *B. pyocyaneus* EMPYEMA IMMEDIATELY BEFORE THE LOCAL INJECTION OF STREPTOCOCCAL CONCENTRATE

The nuclei of the leucocytes, and many extracellular granules and fibrous strands take the Feulgen stain. $\times 882$.

into patients and stained preparations made of serial samples of the exudate, the rapid and complete disappearance of the extracellular material has been observed. The details of such findings in two patients are given in association with the descriptions of the accompanying photographs which are illustrative of the rapidity and completeness of the disappearance of the extracellular desoxyribose nucleoprotein at the site, *in vivo*, of the exudation.

Figures 9 and 10 depict results obtained in a patient with relatively recent exudation consisting chiefly of granules and fibrous strands. Figures 11-13 illustrate the type of Feulgen staining material in a more protracted case of empyema. In both instances the injection of streptodornase resulted in complete disappearance of the extracellular desoxyribose nucleoprotein, within one hour in the first patient, and within two hours in the second patient.

bose nucleic acid from calf thymus has been found to have the following characteristics:

- a. A linear relationship between enzyme concentration and depolymerase activity.
- b. Temperature inactivation of the enzyme exceeds the thermal enhancement of reaction velocity above 45° C. Temperature inactivation is apparent above 30° C.
- c. Depolymerase activity occurs over a wide pH range, and is greatest between 7.0 and 8.5.
- d. In addition to depolymerization, there is a progressive liberation of acid soluble P and N from the substrate. The liberated P appears entirely in the organic form.
- e. Salicylate, gentisate, ascorbate, thiourea, paraminobenzoic acid, parachlorophenol, oxalate, colchicine, and penicillin had no signifi-

The extracellular matrix has been swept clean of the Feulgen staining material. $\times 882$.

The leucocytes remaining visible in the photographs taken following the lytic action represent to the greatest extent, cells that have not degenerated and are morphologically undamaged by the nuclease. A progressive fall in the percentage of degenerating cells, as determined by the Feulgen stain, was regularly observed for at least 24 hours after the injection of the streptococcal concentrate. As shown in Figure 14 the effect is greater, the larger the percentage of degenerating cells in the exudate prior to the injection of the concentrates.

The effect of streptodornase on motility and other functions of leucocytes will be the subject of a separate communication.

SUMMARY

1. The action of streptodornase contained in the streptococcal concentrates on purified desoxyri-

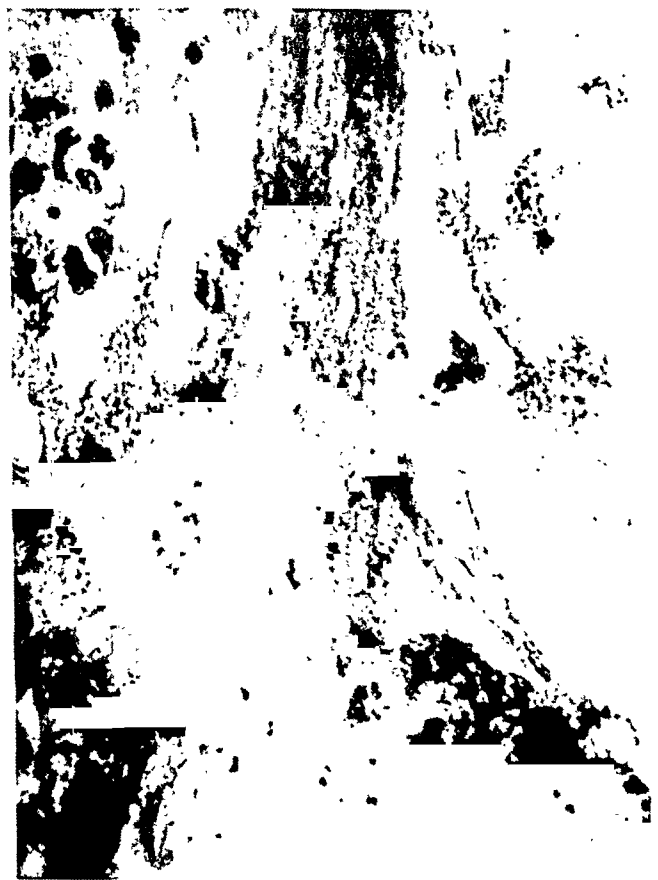


FIG. 11. FEULGEN STAIN OF PLEURAL EXUDATE ASPIRATED FROM A PATIENT WITH A THICK TUBERCULOUS EMPYEMA, IMMEDIATELY BEFORE THE LOCAL INJECTION OF STREPTOCOCCAL CONCENTRATE

The nuclei of the leucocytes, and extensive extracellular amorphous plaques and fibrous strands take the Feulgen stain. $\times 882$.

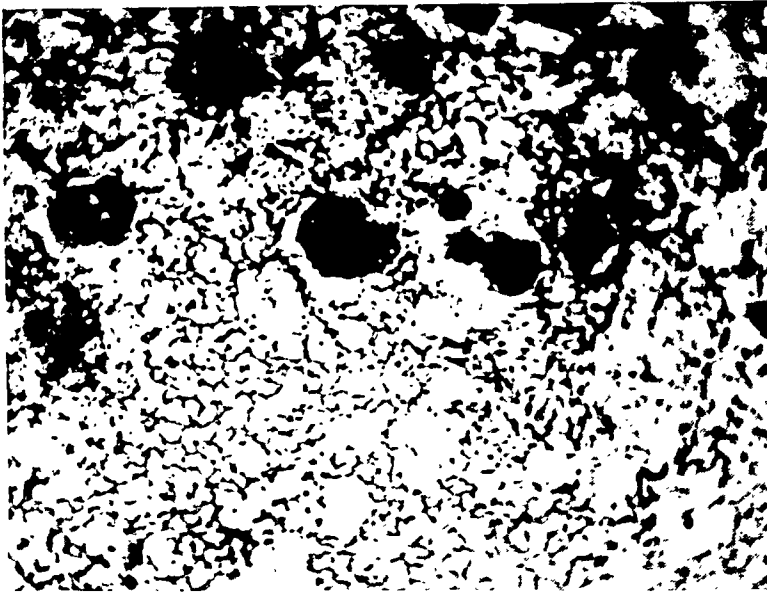


FIG. 12. FEULGEN STAIN OF PLEURAL EXUDATE ASPIRATED FROM THE PATIENT WITH A THICK TUBERCULOUS EMPYEMA, ONE HOUR AFTER THE LOCAL INJECTION OF STREPTOCOCCAL CONCENTRATE CONTAINING 7200 UNITS OF STREPTODORNASE

The amorphous plaques and fibrous strands have begun to break up and disappear. $\times 882$.



FIG. 13. FEULGEN STAIN OF PLEURAL EXUDATE ASPIRATED FROM THE PATIENT WITH TUBERCULOUS EMPYEMA, TWO HOURS AFTER THE LOCAL INJECTION OF STREPTOCOCCAL CONCENTRATE

The extracellular matrix has been swept clean of the Feulgen staining material. $\times 882$.

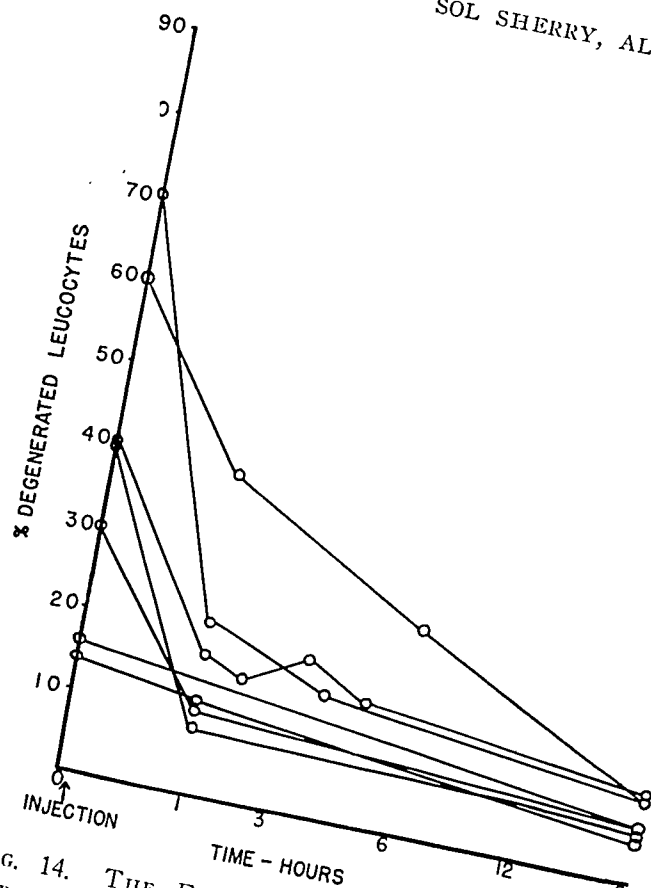


FIG. 14. THE EFFECT OF STREPTODORNASE, INTRA-
PLEURALLY INJECTED, ON THE PERCENTAGE OF DEGEN-
ERATED LEUCOCYTES OF THE PLEURAL EXUDATE IN SEVEN
PATIENTS WITH EMPYEMA

cant inhibitory effect on depolymerase activity. Citrate and heparin were found to have an inhibitory action.

2. Streptodornase has been found to be operative when introduced locally into the pleural cavity of patients with empyema by effecting the rapid depolymerization of the desoxyribose nucleoprotein that has been found to constitute a considerable proportion of the solid sediment of coarse purulent exudates.

The following physical, chemical, histological, and cytological changes have been described in this article.

- Grossly visible changes of the coarse sediment to a thin milky type of fluid.
- Striking fall in viscosity.
- Striking fall in per cent sediment.
- Sharp rise in organic acid soluble phosphorus and acid soluble nitrogen.
- No rise in inorganic phosphorus.
- No increase in uric acid.
- Striking decrease in number of degenerated leucocytes.

h. Rapid disappearance of all extracellular Feulgen staining material. (desoxyribose nucleoprotein).

3. The changes mentioned above were demonstrable in a significant degree within one hour following the injection of streptodornase. Following a single dose, they continued for varying periods of time ranging from several hours to several days, but were self terminating.

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STUDIES WITH RADIOACTIVE IODIZED FAT. I. PREPARATION OF RADIOACTIVE FAT WITH OBSERVATIONS ON THE ABSORPTION OF FAT FOLLOWING SUBCUTANEOUS AND INTRAPERITONEAL INJECTION IN DOGS¹

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INTRODUCTION

The intravenous administration of emulsified fat in concentrations suitable for nutritional purposes is not applicable for clinical use because an emulsion stabilizer free of undesirable effects has not yet been developed. Pending the availability of a satisfactory stabilizer, the possibility of parenteral administration of fat by other routes deserves further exploration. Data reported on the absorption and distribution of fat given subcutaneously, intramuscularly and intraperitoneally to animals (1-6) are equivocal or conflicting largely because of the inadequacy of the quantitative analytical methods used. Fat labelled with a radioactive element can be traced and measured with considerable accuracy. While fat labelled with radioactive carbon (C^{14}) (7) or deuterium (8) is ideal, the measurement in tissue is more difficult than that of fat labelled with radioactive iodine. Although iodized fat is unnatural, its digestion, absorption and metabolic fate after oral and parenteral administration is said to simulate closely that of natural fat (9-15). Since iodine is liberated relatively slowly from iodized fat (16), it should, if radioactive, be useful to follow the absorption and immediate distribution of fat, once it enters the circulation. Accordingly, an emulsion of fat labelled with radioactive iodine was prepared with Demal 14² in order to determine with reasonable accuracy, (1) the absorption and distribution of fat introduced subcutaneously or intraperitoneally, and (2) the distribution of radioactive fat emulsions introduced intravenously. This communication presents a method for the preparation of radioactive fat, to-

gether with data on the absorption of such fat from the subcutaneous and intraperitoneal spaces, with and without hyaluronidase. In a subsequent paper (16), observations on the distribution and fate of radioactive fat emulsions injected intravenously are presented.

EXPERIMENTAL METHODS

Iodination of soya bean oil

A modification of the method for iodination of vegetable oil with iodine monochloride (17) was used for iodination of soya bean oil with $I^{131}Cl$. By this method only one atom of iodine is incorporated upon halogenation of an ethylenic linkage. By using an excess of oil an average of 0.46 atoms of iodine was incorporated into each molecule of fat.³

Potassium iodide (3.6 gms.) was dissolved in 6.2 cc. of distilled water, to which one to two molecules of carrier-free I^{131} (1 cc.) and 2.4 gms. of potassium iodate were added. Concentrated hydrochloric acid (7.2 cc.) was added slowly with vigorous shaking and stirring. The resulting heavy dark precipitate of $I^{131}Cl$ slowly redissolved. A small amount of iodine vapor appeared in the reaction flask during the procedure. The final solution was clear and orange colored. A highly diluted aliquot was taken for radioactivity measurement.

The $I^{131}Cl$ solution was cooled to 4-5° C. and added in three portions with stirring to a cold solution of 50-55 gms. of soya bean oil in 100 cc. ether. The mixture was mechanically shaken for 60-90 minutes at room temperature. The color of the aqueous solution was discharged during this process. The oil-ether solution was straw yellow. It was washed once with water and twice with 5% sodium sulfite in 0.1 N sodium hydroxide solution. It was then washed with water, dilute hydrochloric acid, and finally several times with distilled water. The combined washings contained 2% of the total radioactivity used in the iodination.

³ Pure refined soya bean oil was supplied by E. F. Drew & Co., Inc., Boston. It contains 33.7% oleic acid, 52.0% linoleic acid, 2.3% linolenic acid and 11.2% palmitic and stearic acids. The molecular weight of this oil is about 876 and each molecule contains approximately 4.3 double bonds.

¹ Aided by a grant from the Patrons of Research, Beth Israel Hospital, Boston.

² A preparation of polyglycerol esters provided through the courtesy of Emulsol Corp., Chicago.

The ether solution was dried with anhydrous sodium sulfate and the ether was removed by distillation under nitrogen at reduced pressure and at a temperature between 40–50° C. The residual oil was pale yellow. No radioactivity due to inorganic iodine could be extracted from the oil with sodium sulfite solution after standing in the dark for ten days. The yield was 56 gms. In several runs 80–90% of the iodine used was incorporated in the fat, as determined both by radioactivity measurements and iodine titration.

A 10% emulsion of radioactive oil in water was made with the aid of "Demal 14" as follows: One part of Demal was dissolved in two parts of oil, water was added and the entire mixture was blended for 10 minutes in a Waring blender and then recirculated in a high pressure homogenizer for 60 minutes. Microscopic examination revealed the larger fat droplets to be approximately 0.5–2.0 micra in diameter.⁴

Radioactivity determination (18, 19)

Either 2 cc. of blood or 1 cc. of plasma was evaporated for 48 hours at 37° C. to dryness in an aluminum cup, 2 cm. in diameter. In experiments in which tissues were analyzed, several one-gram specimens of liver, spleen, lung, mesenteric fat, omentum, kidney, intestines, and thyroid were removed in thin slices, immediately after death by exsanguination, wiped dry of all excess blood, and rapidly weighed on a torsion balance. They were then spread out in a thin layer in aluminum cups and evaporated for 48 hours at 37° C. to dryness.

A two to four hundredfold dilution of the radioactive oil or emulsion which was used for injection was prepared with non-radioactive oil or emulsion. An aliquot of this solution was evaporated with 2 cc. of blood or 1 cc. of plasma from a normal dog in order to prepare a standard approximating the dry weight of the experimental samples.

Radioactivity measurements were made on a Tracerlab Autoscaler with a Victoreen Geiger tube (bell type with end mica window weighing 3.6 gms./cm.²). The tube and samples were shielded with 1.5 inches of lead. All specimens were at least five times background, unless otherwise indicated.

Radioactivity measurements were calculated for total activity circulating in the blood or plasma in per cent of total activity injected in the form of oil or emulsion. Tissue radioactivity was calculated in terms of radioactivity per whole weighed organ or tissue in per cent of total activity injected. The standards prepared with normal dog blood were used in making these calculations. For the purpose of these calculations the skeletal muscle was assumed to have 30% (20) and the blood volume 10% of the body weight. The hematocrit was taken as 50%.

⁴ Acknowledgment is due the Esselen Research Corporation, Boston, for assistance in the preparation of several of the early emulsions.

ANIMAL EXPERIMENTS

Subcutaneous injection of radioactive iodized oil

Ten cc. of radioactive oil were injected into the subcutaneous tissues of the flank in each of two dogs. Just prior to the injection of the oil, one of these dogs was also given 20 mgs. of hyaluronidase⁵ in 25 cc. of saline into the same site. In both dogs the injected tissues were massaged to disperse the injected material.

The rate of absorption of radioactive fat into the circulation was determined by measuring serial blood samples for radioactivity. At no time in the following 48 hours was more than 0.25% of the total radioactivity injected found in the circulating blood (Table I). After four to five days the sites of injection retained virtually all of the oil still unabsorbed.

TABLE I
Radioactivity* circulating in the blood† of dogs after the subcutaneous injection of 10 cc. radioactive iodized soya bean oil

Dog no.	Dose of hyaluronidase	Hours after injection								
		1	4	6	8	10	24	28	48	72
1	mgs. 0	—	—	—	0.14	0.16	0.18	0.2	0.14	—
2	20	.08	0.14	0.10	0.10	0.25	0.22	—	—	0.30

* Radioactivity expressed in per cent of total radioactivity injected.

† Blood volume estimated as 10% of body weight.

Ten cc. per kilogram of body weight of the radioactive fat emulsion were injected subcutaneously into each of four dogs. In three of these dogs 5–10 mgs. of hyaluronidase were injected into the same site just before injection of the emulsion. The tissues were massaged following injection of the emulsion. The results were similar to those with liquid radioactive oil. At no time during the following week was more than 0.5% of the total radioactivity injected found in the circulating blood (Table II). Analysis for radioactivity of the major tissues three to five days after injection

⁵ Hyaluronidase is said to increase the rate of absorption of substances injected subcutaneously (21–24) by virtue of the increased surface area made available by its hydrolytic action on the hyaluronic acid gel of the connective tissues. Hyaluronidase was provided through the courtesy of Schering Corp. The assay of each milligram was 50 turbidity reducing units.

TABLE II

Radioactivity circulating in the blood† of dogs after the subcutaneous injection of radioactive oil emulsion*

Dog no.	Dose of hyaluronidase	Hours after injection																
		1	3	4-5	8	12	16-18	23-24	28	33-36	48	54	72-75	90	120	140	160	180
	mgs.																	
3	10	0	0.07	0.04	0.10	0.22		0.23										
4	5	0.02	0.01	0.03	0.06	0.11		0.45		0.48	0.39	0.24	0.12					
6	10		0.04			0.08	0.04	0.07	0.12	0.10	0.14	0.11	0.12	0.07	0.13	0.20	0.25	0.23
7	0		0.02			0.05	0.03	0.06	0.07	0.06	0.14	0.16	0.25	0.33	0.28			

* Radioactivity expressed in per cent of total radioactivity injected.

† Blood volume estimated to be 10% of body weight.

revealed that less than 0.5% of the total radioactivity injected was present in all the tissues combined. The thyroid gland also contained the same low order of radioactivity as other tissues. Only a trace of radioactivity was found in the feces and urine. The injection site contained nearly all of the emulsion. Therefore, neither emulsification nor hyaluronidase improved the absorption of radioactive fat from the subcutaneous tissues.

Intraperitoneal injection of radioactive iodized oil

Ten cc. per kilogram body weight of radioactive fat emulsion were injected intraperitoneally into six dogs. In five of these dogs (Nos. 9, 11-14), 5-10 mgs. of hyaluronidase in 50 cc. of saline were injected intraperitoneally five to 10 minutes prior to the administration of the oil. The results are summarized in Table III.

In Dog 10, which did not receive hyaluronidase, less than 0.5% of the total radioactivity injected was found in the circulating plasma. The same was true in Dogs 11, 12, and 14, which did receive hyaluronidase. Dog 11 was first given the

fat emulsion without hyaluronidase; but six weeks later, he received it again with hyaluronidase. The circulating radioactivity was not increased by the hyaluronidase.

In Dogs 9 and 12, by the 12th day after injection, 8% and 3% respectively of the total radioactivity injected was found in the circulating plasma.

The tissues of Dogs 10, 12, 13 and 14, sacrificed 12-15 days after injection, failed to reveal any radioactivity, except in the peritoneal cavity, which was free of any noticeable reaction to the presence of the fat or hyaluronidase.

DISCUSSION

Our finding of a low order of radioactivity in plasma and tissues, including the thyroid, confirm the view that the subcutaneous route is unsatisfactory for the parenteral administration of fat or fat emulsion. The absorption is too slow and is not noticeably accelerated by hyaluronidase, which does speed up the absorption of water, electrolytes, and plasma (24, 25).

TABLE III

Radioactivity circulating in the plasma† of dogs after the intraperitoneal injection of radioactive oil emulsion*

Dog no.	Dose of hyaluro- nidase	Days after injection																				Wt. loss or gain by the 14th day
		0.17	0.5	1	2	2.6	3	4	5	6	7	7.5	8	9	12	13	14	16	17	21	26	
	mgs.																					lbs.
9	5	0.27	0.24	0.24	1.5		2.9	3.2		5.5	4.5		5.2	7.2	8.1	7.7	7.4		6.3	7.2	0.2	-8
10	0	0.06	0.07	0.08	0.14		0.16	0.16		0.16	0.26		0.21	0.22	0.06	0.04						+0.5
11	0	0.09	0.08	0.14	0.27		0.46	0.72	0.76	0.36	0.44					1.38			1.02	0.46	0.21	-3.5
11	5			0.08	0.11		0.17	0.14	0.17	0.13		0.13	0.2				0.1					+2
12	5			0.19	0.40		0.75	0.52	0.43			1.35			3.26	2.7		1.6				-7
13	10			0.08		0.33		0.43	0.31	0.24			0.19		0.11							0
14	5			0.04	0.05		0.05	0.06	0.07	0.11		0.12		0.17	0.12		0.09					+2

* Radioactivity is expressed in per cent of total radioactivity injected.

† Plasma volume estimated to be 5% of the body weight.

Absorption of fat from the peritoneal cavity is also unsatisfactory. In two of the five dogs which received hyaluronidase, a relatively high level of circulating radioactivity was present from the third day up to the 21st (Dog 9), and the 16th (Dog 12) day respectively following injection of the emulsion. Since injected ionic iodine is rapidly excreted (18), the continuous presence of radioactive iodine in the plasma of these dogs is due either to the continuous slow absorption of the iodized fat or to slow liberation of I^{131} from the iodized fat *in situ*. How much of the circulating radioactivity was ionic iodine cannot be stated. In another publication (16), data are presented showing that 24 hours after intravenous injection of emulsified radioactive fat, only 10% of the radioactivity in plasma is in the form of ionic iodine, while 60% is in the lipid fraction. We did not make this fractional determination in Dogs 9 and 12, but one might postulate a similar distribution in these dogs. A possible relationship between the nutritional state of the animal and the rate of absorption from the peritoneal cavity is suggested, since there was a weight loss of 7-8 lbs. in the course of the experiment in Dogs 9 and 12, whereas Dogs 10, 13, and 14 either maintained or gained weight. Dog 11 showed greater absorption in the second experiment, in which there was a weight loss of 3.5 lbs., than in the first experiment in which there was a weight gain of 2 lbs.

Although virtually all the fat injected intraperitoneally remained there at least 16-17 days, intraperitoneal adhesions did not form in dogs which received hyaluronidase. Since adhesions have been observed from intraperitoneally injected fat without hyaluronidase (5), these observations suggest that this enzyme may be useful to prevent adhesions.

CONCLUSIONS

A method for preparing radioactive iodized oil is described.

Following subcutaneous or intraperitoneal injection of emulsions of this oil, absorption was slow and nearly all of it remained at the site of injection.

Hyaluronidase did not increase the rate of absorption of tagged fat from the subcutaneous or peritoneal space.

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STUDIES WITH RADIOACTIVE IODIZED FAT. II. THE TISSUE DISTRIBUTION OF EMULSIFIED FAT FOLLOWING INTRAVENOUS ADMINISTRATION¹

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The rate of absorption of emulsified fat labelled with radioactive iodine from the subcutaneous tissues and the peritoneal cavity in animals is too slow to serve as a source of current caloric requirements (1). Since parenteral administration to patients for this purpose would require that the fat be given in high concentration intravenously, it is necessary to prepare a stable, non-toxic emulsion. This has not yet been accomplished (2-7). Pending the availability of such an emulsion, it is desirable to study the metabolic fate of fat given intravenously. One of the first steps in such a study is the distribution of the injected fat. Numerous studies of fat, tagged with non-radioactive iodine, given by vein, fail to agree as to its distribution (8-19). The conflicting results are in part due to variations in particle size and to lack of reliable techniques for determining tissue localization and quantitative distribution.

In this communication, we report the quantitative tissue distribution of the radioactivity found after the intravenous injection into dogs and mice of an emulsion of radioactive iodized soya bean oil. The oil was labelled with radioactive iodine by a technique reported in a preceding paper (1). It was then prepared as an emulsion as follows:

Preparation of the emulsion

Radioactive iodized soya bean oil (1) was emulsified in water with the aid of "Demal 14."² Twenty-five gms. of this detergent were dissolved in 50 gms. of the oil, and the resulting solution was warmed to 80° C. and slowly added to 425 cc. of warm distilled water in a rotating Waring blender. The blending was continued for ten minutes after the oil mixture had been added. The resulting crude emulsion (10%) was then homogenized by recirculation for 60-90 minutes in a two-stage

homogenizer at 3300-3500 lbs. of pressure per square inch. The temperature rose to 70-85° C. The final product appeared blue by reflected light and reddish by transmitted light, an indication of fine particle size (20). On microscopic examination the oil globules ranged from approximately 0.5 to 2.0 micra in diameter. The emulsion was stable at room temperature for one to two weeks. Since sterilization by autoclaving destroyed the emulsion and decreased its pH from 8.0 to 4.3, it was passed through a Seitz filter before use. The emulsion was found apparently non-toxic and well tolerated when given orally and intravenously to mice and dogs.

ANIMAL EXPERIMENTS

Intravenous administration in dogs

Each of eight mongrel dogs (weight range 8-12 kgs.) was given a single slow intravenous injection (1.2-4.2 cc./kg.) of the emulsion. Venous blood samples were drawn at intervals. Two cc. of blood or 1 cc. of plasma was used for radioactivity measurements. Standards consisted of highly diluted aliquots of the emulsion, to which 2 cc. of normal blood or 1 cc. of normal plasma was added. The technique of radioactivity measurement has been described elsewhere (1).

The results, listed in Table I, show that at the end of the first hour following injection about 2% of the total injected radioactivity was circulating in the blood of Dog 8, and about 1% in the plasma of Dogs 8, 20-24. There followed a slow increase, so that some 24 hours later the maximum concentration of circulating radioactivity was reached (some 3% to 7.5% of the total injected radioactivity). This maximum, with some variation, persisted for another 24-48 hours and gradually declined thereafter.

Plasma Fractionation Experiments

The distribution of the circulating radioactivity among the plasma components was examined by fractionation of plasma specimens drawn from several dogs 24-30 hours after injection of the emulsion.

¹ Aided by a grant from the Patrons of Research, Beth Israel Hospital, Boston.

² A blend of di-tri and higher polyglycerols, partially esterified with oleic acid. Supplied by the Emulsol Corp., Chicago, Illinois.

TABLE I

Circulating radioactivity at various intervals after injection of radioactive fat emulsion in dogs*

Dog no.	8	8	20	21	23	24	22
Specimen	Blood	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma
Volume emulsion injected cc./kg.	4.2	4.2	1.2	2.5	3.1	3.2	3.8
Hours after injection	Circulating radioactivity in per cent of total activity injected						
0.25				0.8			1.2
0.50				1.4			0.9
0.75	2.0	0.7	1.8				
1			0.9				
2			1.1	1.1			1.2
3	1.9	1.1	1.6				
5	2.3	1.3	2.0	1.1			1.7
7	2.2	1.4	2.1	1.6		1.3	2.6
8	2.0	1.2					
12			3.6	3.8			2.4
20	2.2	1.5	4.2	2.4			3.0
24-26	4.5	2.7				3.5	
28-30	5.5	3.2	7.4	7.0	6.5		6.1
45-54	4.6	2.3	7.5			3.0	
63	4.4	1.9	6.3	10.5	7.8	2.9	
68-72	3.6	2.0					
84-88	3.1	1.7	4.3	12.4	7.0		
102		1.7	2.6				
112		1.5					
120			0.7				
170				9.0	6.7		
216					5.2		
243					4.2		
252					1.2		
					0.2		

* Assuming blood volume to be 10% and plasma volume to be 5% of body weight.

The isolated fractions or their aliquots were transferred to aluminum cups (2 cm. in diameter), evaporated to dryness and the radioactivity measured (1). The results were calculated in per cent of the radioactivity of the plasma which was fractionated.

Four types of fractionation experiments were performed as follows:

Experiment 1.—Analysis of lipid fractions of plasma by alcohol-ether extraction

Total lipids and phospholipids were isolated from the plasma according to the method of Bloor (21). The plasma proteins precipitated by the alcohol-ether mixture were also collected. The total protein and phospholipid fractions contained only traces of radioactivity, while the total lipids contained 50-65% of the radioactivity.

Experiment 2.—Analysis of the alcohol-ether extract of plasma

An alcohol-ether (lipoid) extract of plasma was evaporated to dryness and the lipoids in the residue redissolved in hot petroleum ether. The petroleum ether extract contained 60% of the total radioactivity of plasma.

The residue, after lipid extraction, was redissolved in water. An aliquot of this solution showed that it contained 20% of the total radioactivity of whole plasma.

The aqueous solution remaining after extraction with alcohol-ether was acidified with nitric acid. Carrier sodium iodide and a solution of silver nitrate were added in that order. The yellow silver halide precipitate was collected, washed thoroughly with water, acetone and ether. The radioactivity of this precipitate (*i.e.*, ionic iodine) was 9-12% of the total radioactivity (standard prepared with the same quantity of silver halide).

Experiment 3.—Further analysis of the alcohol-ether extract of plasma

A sample of plasma was extracted with alcohol-ether. The extract was divided into two portions. These were treated as follows:

(a) Isolation of ionic iodine from alcohol-ether extract:

To one portion nitric acid, carrier sodium iodide, and silver nitrate in aqueous solution were added in that order. The yellow precipitate was collected, dried, and washed thoroughly with ether and petroleum ether. The halide fraction contained 25-30% of the radioactivity of the alcohol-ether extract.

(b) Isolation of ionic iodine from lipid-free alcohol-ether extract:

Another portion was evaporated to dryness and extracted with water. Silver halides precipitated from the extract contained approximately 10% of the total activity.

Experiment 4.—Analysis of the protein and lipid-free filtrate of plasma

Protein was precipitated with sodium tungstate from an aliquot of plasma by the method of Folin and Wu (21). The filtrate was evaporated to dryness, extracted with ether and the residue dissolved in water and filtered. Ionic iodine was precipitated with silver nitrate as above. The precipitate of silver chloride and silver iodide was collected, washed thoroughly with water, acetone, and ether, and its radioactivity determined. The protein precipitate had no measurable radioactivity. The silver precipitate (*i.e.*, presumably the ionic iodine fraction) contained 15-20% of the activity in plasma.

The results for fractions extracted by organic solvents are somewhat high (error approximately 15%) and those for the silver halide precipitates somewhat low (error approximately 10-20%), because of differences in the dry weights of these samples as compared with standard plasma specimens. Corrections were not made for such variations in weight except to determine the per cent of error given above by suitable control experiments.

These experiments (Table II) show that 24 hours after the injection of the emulsion approximately 60% of the circulating plasma radioactivity was in the total lipid fraction and approximately

TABLE II
Radioactivity circulating in fractions of plasma of dogs
24 hours after injection of radioactive fat emulsion,
expressed in per cent of the activity
in the circulating plasma

Experi- ment no.*	Per cent of radioactivity in circulating plasma				
	Lipoid fraction	Inorganic fraction		Protein	Phospho- lipid
		Precipitated with silver	Insoluble in organic sol- vents but soluble in H ₂ O		
1	%	%	%	%	%
2	50-65	9-12†	20	0	0
3a	60	25-30†			
3b		10			
4		15-20†		0	

* The results listed for each type of experiment were obtained from two to four experiments.
† These samples contained silver chloride in addition to silver iodide.
‡ These samples may have contained the silver salt of radioactive fatty acid.

20% was in the water soluble residue, free of lipid and protein (Experiments 2 and 4). The value, 9-12%, is a low estimate of what was present as ionic iodine (Experiment 3b). In a pilot experiment this discrepancy of 11-8% was accounted for by the self-absorption of β -rays by the heavy mass of the silver precipitate. Part of

the 25-30% radioactivity in the silver halides precipitated from an alcohol-ether extract (Experiment 3a) may be due to radioactive fatty acids carried down with the halides.

Tissue Distribution of Radioactivity After Injection of Fat Emulsion

A. In Dogs

Tissue analyses for radioactivity by methods previously described (1) were performed in five dogs sacrificed by exsanguination at various intervals after injection of the emulsion.

The results, listed in Table III, are expressed in (1) activity per organ in per cent of the total activity injected, and (2) activity of 1 gm. of tissue in per cent of activity circulating at zero time in 1 cc. of plasma, assuming the plasma volume of each dog to be 5% of the body weight.

Samples (1 gm.) of various tissues when wet did not yield dry tissues of uniform weight. Error was thus introduced into the radioactivity measurements by difference in self-absorption of β -rays. However, the dry weight of any given tissue from one animal to another was remarkably uniform and changes in the radioactivity of any given tissue with time could be reliably compared (22).

Of the tissues of Dog 22, sacrificed one day after

TABLE III
Total* and specific† radioactivity in tissues of dogs at various intervals after injection of radioactive fat emulsion

Dog no.	22		24		21		23		20	
	1		2		5		10		25	
Interval from injection to death (days)	3.8		3.2		2.5		3.1		1.2	
Volume of emulsion injected (cc./kg.)										
Tissue	Total*	Specific†	Total*	Specific†	Total*	Specific†	Total*	Specific†	Total*	Specific†
Plasma	6.1	6.1	2.8	2.8	9.0	9.0	0.2	0.2	0.2	0.3
Lung	9.9	73.8	2.9	19.2	0.5	3.0	0.06	0.3	0.02	0.6
Liver	15.6	35.2	4.7	6.2	0.5	2.2	0.3	0.4	0.3	0
Spleen	4.0	93.4	1.5	28.4	0.5	11.0	0.05	1.1	0.3	0.3
Kidneys	0.3	3.4	0.2	2.5	0.5	4.6	0.03	0.2	0.1	0.1
Intestines	0.7	1.4	0.9	0.8	0.05	1.5	0.04	0.2	0.1	0.1
Mesenteric fat	0.1	0.5	0.2	0.5	0.7	0.6	0.9	0.3	0.1	0.1
Muscle	4.7	0.7	4.1	0.1	3.9	0.7	0.1	87.7	0.1	89.2
Thyroid	0.02	9.6	0.04	60.7	0.3	123.5	0.04	0.1	0.3	
Bile	0.04	1.5	0.04	2.2	0.02	0.8	0.04	0.1	0.52	
Total	41.46	17.44		17.07		1.92				

* Expressed in per cent of total radioactivity injected. Muscle assumed to be 30% of body weight (23). All other organs weighed wet.
† Radioactivity of 1 gm. tissue, expressed in per cent of the radioactivity circulating at zero time in 1 cc. plasma, assuming plasma volume to be 5% of total body weight.

injection, the liver, lungs, plasma, muscle and spleen, in decreasing order, contained the largest fractions of the total radioactivity. Mesenteric fat and thyroid showed the lowest radioactivity content of all tissues examined. Some 41% of the total radioactivity injected was recovered in the tissues examined.

In Dog 24, sacrificed two days after injection, only 17% of the total radioactivity injected was recovered in these same tissues. The same amount was recovered from the same tissues in Dog 21, sacrificed five days after injection. In Dog 23, sacrificed ten days after injection, only some 2% of the amount injected was found in these tissues.

The spleen, lungs and liver, in decreasing order, showed the highest specific radioactivity one day after injection (Dog 22). The same was true after two days (Dog 24) although a substantial decline was obvious in all these organs, while the thyroid showed a very large increase in specific activity. This was all the more striking after five days (Dog 21). The spleen exhibited the slowest decline in specific activity. The continuously very low total and specific radioactivity of

mesenteric fat is noteworthy. This is all the more so, in view of the fact that every other tissue studied showed a higher specific activity, at least during the first five days after injection. Ten days after injection very little activity was found in any of the tissues except the thyroid.

Specimens of urine were collected at various intervals after injection. The total urine collected in one of the dogs (Dog 20) during the first eight hours following injection contained 2½% of the total activity injected. By the third day, the specific³ activity of the urine averaged 0.5% in all dogs. It remained at that level for the duration of the experiment in Dogs 20, 21 and 23.

B. In Mice

White stock mice, injected with 0.25 cc. of emulsion via a tail vein, were killed by decapitation at specified intervals. Blood collected in heparin during decapitation, and various tissue specimens taken immediately thereafter, were examined for radioactivity. The specific activity of each tissue,

³ Radioactivity in 1 cc. of urine, expressed in per cent of the activity circulating at zero time in 1 cc. of plasma.

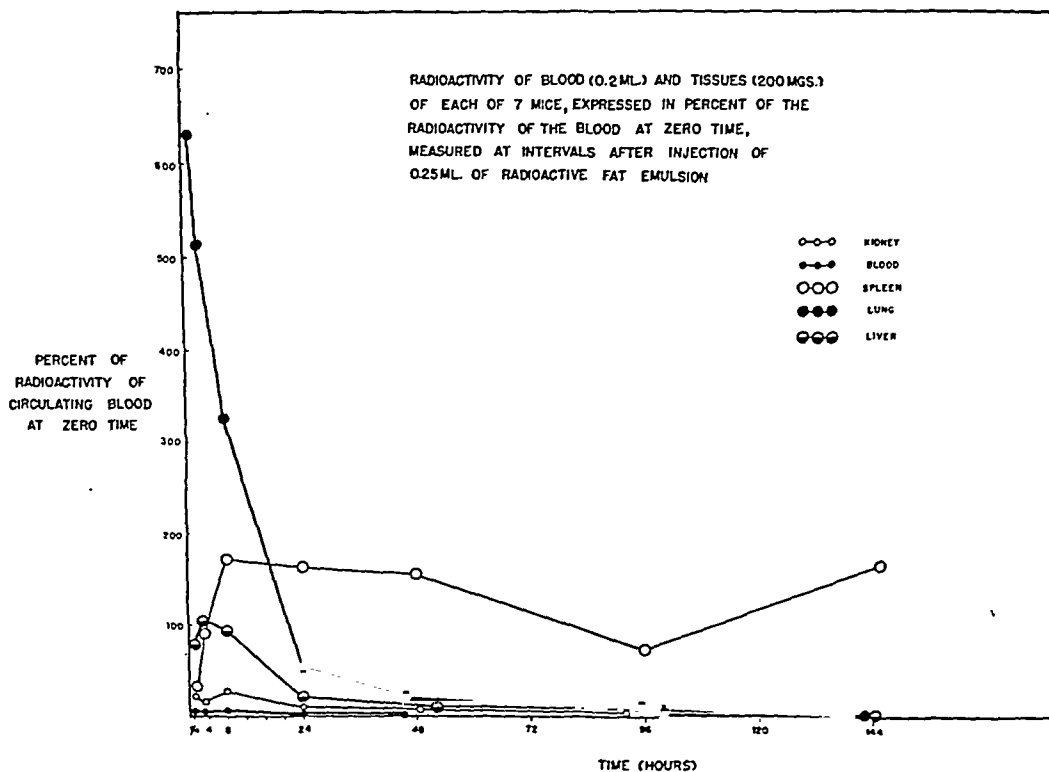


Fig. 1

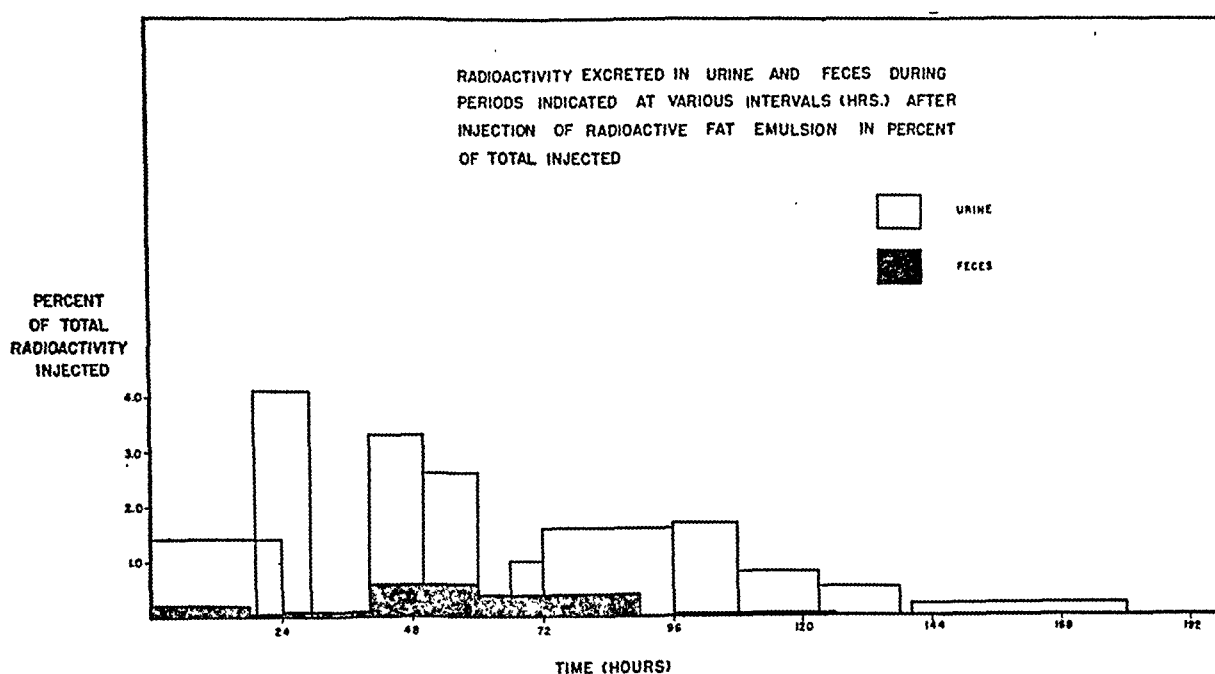


FIG. 2

in per cent of the activity circulating at zero time, was determined by a technique previously described (1, 22, 24). This experiment was performed in three groups of eight to 12 mice each.

The results of a representative distribution study (Figure 1), calculated according to methods reported elsewhere (22) are expressed in per cent of total activity injected. Errors in radioactivity determinations due to differences in dry weights of different tissues were not corrected, but the dry weight of the same tissue in different animals was remarkably uniform (22).

Following injection, the radioactivity disappeared rapidly from the blood. Three hours after injection, lung, liver, and spleen showed high levels of specific radioactivity, that of lung being extraordinarily high.

Twenty-four hours after injection, however, the level of radioactivity was highest in spleen, next highest in lung, and next in liver. The level in spleen increased thereafter, and continued at a high level for six to eight days, but that in liver and lung slowly declined. The specific activity in thyroid was high, but the cumulative increase with time, observed in the dog, did not occur. Intestine and fat showed a very low level of activity. Brain, muscle and skin showed no measurable activity.

In another group of some 20 male mice, similarly injected, specimens of urine and feces were collected at various intervals after injection and

measured for radioactivity. The urine specimens were obtained by aspiration of the exposed bladder of these mice, sacrificed ten to 24 hours following ligature obstruction of the penile urethra (22). This was done at varying intervals from zero to 140 hours after injection. The urine and feces were transferred to aluminum cups for evaporation to dryness and radioactivity determination (Figure 2). The radioactivity in urine, which was still considerable four to five days after injection (Figure 2), as already stated above for dogs, was presumably in the form of ionic iodine cleaved from the fat. The low activity in the feces may have been due to a small amount of labelled fat excreted into the intestine.

DISCUSSION

Other investigators (8-19) have studied the immediate distribution of intravenously injected, non-radioactive, iodized fat emulsion in dogs and rabbits by determining the total iodine content of various organs. The injected fat left the blood stream rapidly (10, 13) and was retained in part by the lungs (9, 18, 19) or by the liver (8, 11, 12, 14) depending on particle size. Nearly all of an emulsion of greater particle size was deposited in the lungs (14) while most of an emulsion of smaller particle size was deposited in the liver (8, 11, 12, 14) or in the spleen (10, 11). Blockade of the reticulo-endothelial system by colloidal

silver, according to one investigator, resulted in diversion of the fat to the lungs and the liver parenchyma (10), while in similar experiments by others (11) such blockade was said to have had no effect on the distribution of the injected fat, which was reported to be retained by the liver (11).

More reliable indication of the initial distribution of intravenously injected fat is provided by determining the radioactivity of tissues soon after injection of radioactive iodized fat. This is so because the liberation of iodine from the fat molecule is relatively slow, as indicated (1) by the fact that iodine, which appears in the urine as soon as it is liberated, is not found in the urine in any quantity until an hour after the injection of iodized fat, and (2) because the urine shows a continuously high level of radioactivity for five to six days after injection of the emulsion. The metabolic cleavage of iodine from the fat molecule therefore appears to take place gradually and continuously.

From our data, it is not possible to determine the rate of metabolism of the iodinated or deiodinated fat. But the data on specific activity of various tissues are interesting in respect to this question (Table III). The very high specific activity in liver, lung and spleen 24 hours after injection probably reflects the storage function of the reticulo-endothelial system for particulate matter. Comparison of these data from Dog 24 with those of Dog 21 suggest that the spleen and lungs continue to act as depots, steadily, though slowly, giving up their stores, while the liver loses its stores much more rapidly, either by catabolism or by delivery to fat depots. The latter destination of the fat before deiodination has occurred seems unlikely in view of the continuously negligible lack of specific activity of mesenteric fat in five successive experiments. One may hazard the inference that fat depots cannot store any but structurally normal fat or that fat for which there is current need does not participate in the metabolic activity of fat depots. The latter possibility is unlikely in view of Schoenheimer and Rittenberg's study of fat labelled with deuterium (25).

The excretion of radioactivity into the urine, beginning within one hour after injection, represents ionic iodine liberated continuously over a period of several days.

CONCLUSIONS

An emulsion of radioactive iodinated fat injected intravenously in dogs and mice leaves the circulation rapidly.

Approximately 60% of the radioactivity still present in the circulation 24 hours after injection is contained in the lipoid fraction of the plasma and 20% in the ionic iodine fraction.

Immediately after injection, the highest concentration of radioactivity in the tissues of dogs is found in the spleen, lung and liver, in that order, and decreases slowly. In tissues of mice the concentration of radioactivity immediately after injection is highest in the lungs. There it drops rapidly, while the concentration of radioactivity in the spleen increases rapidly and remains high for six to eight days after injection.

Iodine is gradually and slowly liberated from the iodinated fat and excreted. Iodinated fat is not stored in mesenteric fat depots.

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THE MEASUREMENT OF EXTRACELLULAR FLUID IN MAN BY MEANS OF A CONSTANT INFUSION TECHNIQUE¹

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The principle substances in use to date as indicators of the extracellular water volume do not fulfill the basic requirement of exclusive distribution in the extracellular space.

Sodium is normally present in erythrocytes (1-3), striated muscle (4, 5), spinal cord, cartilage and bone (4, 6, 7). Chloride penetrates erythrocytes, connective tissue, pyloric mucosa, testes, salivary glands and cells of the central nervous system (6, 8). Bromide interchanges freely with chloride in all tissues except the central nervous system (9, 10) and therefore probably shares with chloride the property of cell penetration. Thiocyanate is bound to lipid and penetrates erythrocytes, gastric mucosa and other tissues (7, 11-14) and in bacteremia, spotted fever and malaria, and following artificial hyperpyrexia thiocyanate space may approach the value for total body water (15-17). Apparently, therefore, these ions may be used in normal individuals only for approximation of the volume of extracellular water, and in pathological states altered cellular permeability may invalidate even such approximate measurements.

Inulin, sucrose and mannitol are distributed throughout a consistently lower fraction of body water than are the electrolytes (18-23). Although these substances appear to circumvent the problem of cell penetration, they raise the question of how may plasma-interstitial fluid equilibrium be assured in the face of rapid elimination, slow diffusion, or both?

Any substance distributed in some fixed fraction of the body water will approach a uniform or equilibrium concentration throughout that compartment under conditions where its rate of infusion (in milligrams per minute) and its clearance or specific rate of removal (in milligrams per min-

ute per unit plasma concentration) remain constant for a sufficiently long period of time. After uniform distribution is attained, if the infusion is stopped and the total amount of the substance present in the body water is recovered quantitatively in the urine, its volume of distribution is the amount recovered divided by the equilibrium plasma water concentration. The use of inulin in the constant infusion method in dog and man has been reported in a preliminary paper (23), and more definitive data are available in the dog (24). The studies described here were undertaken to supplement our preliminary observations and to develop a standard constant infusion method for the measurement of inulin space in man.

METHODS

Twenty-nine studies were carried out in ten normal male subjects ranging from 21 to 55 years of age, and in five elderly male patients with congestive heart failure² ranging from 45 to 60 years of age. In six of these studies a single intravenous injection of 50 to 75 cc. of 10 per cent inulin was given from a syringe calibrated at 20° centigrade with mercury or water at arbitrary marks. For the next 24 hours samples of blood and serial urine collections were obtained.

In the remaining 24 studies a priming injection of inulin was followed by a constant sustaining infusion for 1.7 to 30 hours. Assuming a distribution volume of 18 per cent of body weight, the prime was calculated to yield a plasma concentration of 10 to 30 mg. per cent and the concentration of sustaining infusion was calculated on an assumed filtration rate. Inulin solutions were made up in sterile isotonic saline except those for the cardiac patients where 5 per cent dextrose in distilled water was used. Constancy of the infusion rate in the first five studies was maintained by adjusting a Murphy drip every five to ten minutes. In all other experiments this was achieved with a constant infusion pump. The rate of

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² The five patients with congestive heart failure were subjects who had been infused with inulin during the course of studies conducted by Dr. David Baldwin, Dr. Herman Villareal, and Dr. Jonas Sirota, who kindly allowed us to utilize these infusions in order to determine the inulin space.

infusion used was 1.3 cc. per minute in all normal subjects and 0.3 cc. per minute in the cardiac patients. Calibration of this pump in successive 15, 30 and 60 minute periods revealed variation of less than 1 per cent at the higher rate and less than 3 per cent at the lower rate.

Varying lengths of infusion were utilized in the same individual whenever possible in order to determine the minimal time for equilibration of plasma water and interstitial fluid and to observe the reproducibility of the measurement of inulin space.

No preparation of the subject was made other than an attempt to maintain adequate and uniform hydration with a urine flow of 2 to 4 cc. per minute during the infusion. Before the priming injection a control blood and timed urine sample were obtained for determination of plasma blank concentration, B_0 , and the rate of urinary excretion of inulinoid blank, U_0V , which quantities were subtracted respectively from subsequent plasma concentrations and from the total inulin excretion during the post-infusion recovery period. Two or more blood samples were taken during the infusions to ascertain constancy of the plasma concentration. Shortly before stopping the infusion, the bladder was catheterized and a final blood sample was drawn. The bladder was washed well with isotonic saline and air. At completion of the wash-out the infusion was discontinued and all subsequent urine was collected in 0.5 to six hour long periods for the next 22 to 48 hours. In the subjects used for repeated studies blood samples were drawn at periodic intervals during this post-infusion period so that the decrement in plasma concentration with time might be compared in the same individual following varying periods of infusion. Blood samples were drawn from an antecubital vein using 0.1 cc. Liquaemin (Hoffmann-La Roche) per 10 cc. of whole blood as an anticoagulant. The patients with congestive heart failure were given 2 cc. of mercupurin intravenously shortly after the infusion was discontinued.

Our present criterion for equilibrium distribution of inulin throughout the extracellular fluid is attainment of a maximal and constant volume of distribution as the duration of infusion is successively increased in the same individual.

In some subjects, thiocyanate, bromide and Na^{24} spaces were also determined by the single injection method. Blood samples were drawn after one hour for thiocyanate and after three to four hours for bromide and Na^{24} determinations. Thiocyanate was determined by the method of Crandall and Anderson (11) adapted to the Beckman Spectrophotometer, model DU. Readings were made at a wave length of 460 $m\mu$ using cuvettes with a light path of 10 mm. Bromide was determined by the method of Brodie and Friedman (25), inulin by Harrison's modification (26) of the method of Alving, Rubin and Miller (27), and Na^{24} was measured with a Geiger-Müller counter. The plasma concentrations of inulin, thiocyanate, bromide and sodium are corrected for a plasma water content of 94 per cent and the concentrations of thiocyanate, bromide, and Na^{24} are corrected for the Donnan factor k : for thiocyanate and bromide, $k = 1.05$ and for

Na^{24} , $k = 0.95$. No correction was made for plasma protein or lipid binding of thiocyanate.

Urines were yeasted and precipitated in the same manner as the plasma. In some cases urines were yeasted both before and after dilution to a U/P ratio of 1, in which case the determinations compared within 2 per cent. Analyses were checked frequently by recoveries of known amounts of inulin added to water and plasma. The pH of the plasma filtrate was kept at or below 7.

The rate of excretion of inulinoid blank, U_0V , was determined in 30 successive one to three hour urine periods for eight to 24 hours in four normal subjects at urine flows varying from 0.4 cc. per minute to 10 cc. per minute, both in the fasting state and during unrestricted dietary intake. Blank excretion showed no relation either to urine flow or time after eating. The average U_0V of 53 periods including the above, was 0.16 mg. per minute with a standard deviation of ± 0.055 mg. per minute. Inulin space errors due to variation in U_0V become less significant as the equilibrium plasma concentration is elevated. In a normal subject with a plasma concentration of 30 mg. per cent and an inulin space of 10 liters, the average error to be expected from variations in U_0V during a 15 hour recovery period is 1.7 per cent, and the error to be expected from complete neglect of U_0V is 4.8 per cent.

RESULTS

Equilibration Time. The inulin volume of distribution was not increased significantly when the infusion was extended beyond 4.5, 3.3, 3.8, 7.5 and seven hours in five normal subjects, respectively, as compared with a minimal infusion period of 1.7 hours (Table I). In one of these subjects determinations were made with infusion periods varying from 1.7 to 17.5 hours. Inulin space increased asymptotically with the duration of infusion approaching a limiting value of 13.7 liters, as calculated by a first order differential equation applied to a curve drawn through the observed points (Figure 1). In this subject the inulin space after five hours of infusion was 95 per cent of the limiting value. The curves of declining plasma concentrations following cessation of infusion at 4.5, 5.5 and 17.5 hours were virtually superimposable when plotted on the same scale (Figure 2). We conclude that for all practical purposes plasma-interstitial fluid equilibrium was established in this subject by five hours.

After a single intravenous injection of inulin the curve of plasma concentration plotted against time shows two phases: an early rapid decrement followed by a period of more gradual decrement.

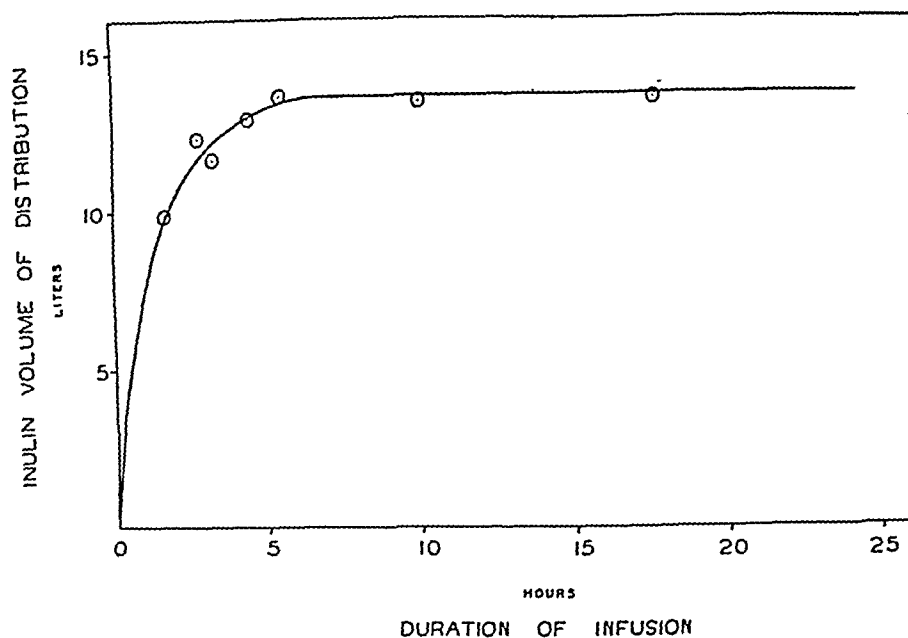


FIG. 1. INULIN VOLUME OF DISTRIBUTION DETERMINED AFTER VARIOUS PERIODS OF INFUSION IN THE SAME INDIVIDUAL

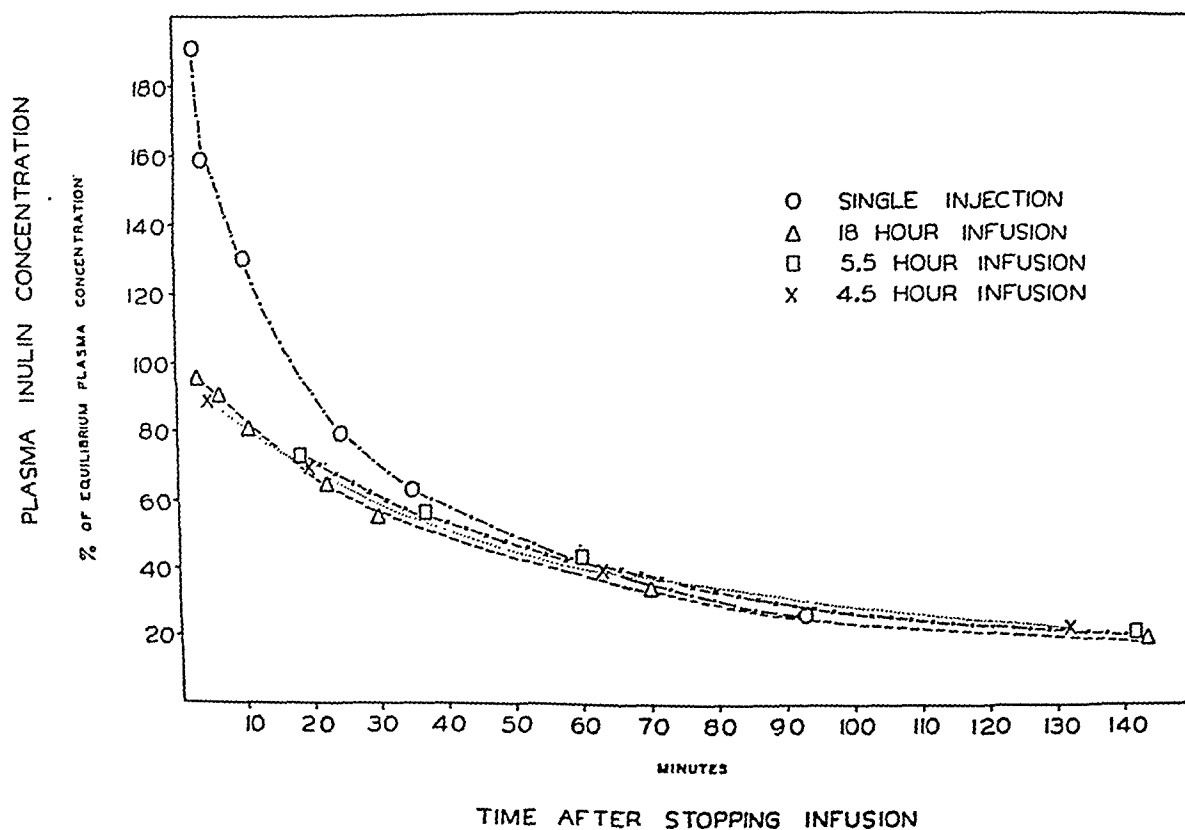


FIG. 2. CONCENTRATION OF INULIN IN PLASMA FOLLOWING A SINGLE INJECTION AND INFUSIONS OF 4.5, 5.5 AND 17.5 HOURS

For the single injection the equilibrium plasma concentration was calculated as the amount of inulin injected divided by the inulin volume of distribution as determined previously by the infusion method.

TABLE I

Volume of distribution of inulin, thiocyanate, bromide and radioactive sodium in man

Subject	Age	Date	Weight	Duration of equilibrating infusion	Equilibrium inulin concentration in plasma water	Duration of urinary recovery	Volume of distribution							
							Inulin	Thiocyanate	Bromide	Radioactive sodium	Percentage of body weight			
											Inulin	Thiocyanate	Bromide	Radioactive sodium
	yrs.		kg.	hours	mg. per cent	hours	liters	liters	liters	liters	per cent	per cent	per cent	per cent
I. S.	29	10/26/48	86.4	1.7	16.0	24	9.9				11.4			
		11/13/48*	86.2	2.8	32.9	24	12.3				14.3			
		8/30/48	87.3	3.3	16.9	23	11.6		21.4	22.7	13.3		24.8	26.4
		9/ 6/48	86.9	4.5	25.8	22	12.9	19.3			14.9	22.2		
		10/16/48	86.5	5.5	24.1	36	13.6				15.8			
		8/24/48	88.5	17.5	13.7	24	13.5				15.3			
D. S.	21	10/22/48	72.7	3.3	18.3	26	12.1				16.6			
		8/28/48	72.2	8.3	22.8	34	12.2	17.4			16.8	24.1		
		8/11/48	72.5	26	16.1	28	12.4				17.1			
N. F.	24	11/ 5/48	73.3	3.8	25.9	24	10.4	15.9	15.1	19.8	14.2	21.6	20.6	27.0
		11/12/48	73.4	7.0	28.5	22	10.9				14.9			
G. F.	51	9/22/48	84.5	4.7	27.1	21.5	12.0				14.2			
		6/24/48	83.5	7.5	22.5	22.5	13.2	18.1	17.8		15.8	21.6	21.3	
		9/29/48	84.6	15.3	22.6	25	13.5	20.7		22.4	16.0	24.5		26.4
M. P.	43	6/17/48	63.2	7.0	8.64	12	10.4	17.0			16.5	26.8		
		6/20/48	63.0	17	18.2	17	10.6				16.8			
D. C.	38	6/29/48	63.7	8.5	10.6	12.4	11.5	16.1			18.0	25.3		
V. S.	50	7/ 7/48	62.3	16.5	4.9	17	10.1	17.2	16.0		16.1	27.5	25.7	
J. T.†	52	5/12/48	53.3	29	10.3	23	16.3				30.5			
F. C.†	49	9/19/48	61.0	26	18.6	31	19.0				31.2			
T. N.†	67	10/ 5/48	62.8	26.5	9.8	48	24.8				39.4			
A. D.†	68	10/19/48	60.0	28	16.4	45	11.5				18.5			
T. P.†	60	11/ 2/48	70	30	8.1	43	15.9				22.7			

* No catheterization, bladder emptied by spontaneous voiding when infusion stopped.

† Patients with congestive heart failure: J. T., Rheumatic heart disease, ++ peripheral edema, enlarged tender liver, moist rales at both lung bases. F. C., Rheumatic heart disease, +++ peripheral edema, enlarged tender liver, right pleural effusion. T. N., Cor pulmonale, ++++ peripheral edema, advanced congestive heart failure. A. D., Arteriosclerotic heart disease, no peripheral edema, dyspneic, orthopneic, depressed breath sounds and moist rales at both lung bases. T. P., Arteriosclerotic heart disease, generalized anasarca on admission to the hospital, digitalized on the ward with good response, + edema on 11/3/48 when infusion stopped.

Two similar phases are seen in the falling curve observed after stopping a constant intravenous infusion, but the initial period of rapid decrement is less prolonged. The duration of this initial phase decreases progressively with increasing durations of infusion until a minimum is reached at equilibration time. Following longer periods of infusion, the curves are identical (Figure 2).

Estimation of equilibration time by serial determinations on the same individual was not feasible in patients with congestive heart failure.

In Patient F. C., who had extensive edema and a right pleural effusion, equilibrium between pleural fluid and plasma was not achieved 20 hours after starting the sustaining infusion. Simultaneous pleural fluid and plasma water inulin concentrations were 13.0 mg. per cent and 17.7 mg. per cent respectively.

Recovery of inulin in urine after single injection. When 5 to 7 gm. of inulin were injected into six normal subjects, urinary recovery accounted for 98.0, 97.6, 91.5, 94.4, 98.7, 101 per cent of the

TABLE II
Quantitative urinary recovery of injected inulin

Subject	Amount of inulin injected	Urine collection		
		Time after injection	Total amount recovered	Total amount injected
	mg.	hours	per cent	per cent
D. S.	7285	1.6	64	98.0
		4.6	87.5	
		8.9	96.5	
		18	99.4	
		20	100	
N. F.	5942	0.5	36.8	97.6
		1.1	52.4	
		1.7	63.1	
		2.2	69.0	
		4.7	84.9	
		22.3	99.8	
		23.4	100	
D. L.	2613	0.6	37.5	91.5
		1.3	53.6	
		1.8	66.9	
		2.5	74	
		4.4	87.6	
		7.3	96	
		10.2	98.6	
		19.4	99	
		25.4	100	
		28.6	100	
I. S.	5340	1.4	65.2	94.4
		2.3	71.8	
		3.4	82.4	
		4.4	87.0	
		6.4	92.0	
		9.4	95.6	
		18.5	98.0	
		26.3	99.0	
		31.4	100	
M. R.	5030	3.3	83.6	98.7
		5.4	92.4	
		13.4	99.2	
		15.8	99.5	
		24	100	
H. L.	6280	24	100	101

amount injected. Over 95 per cent of the total amount recovered was excreted in nine hours (Table II).

Recovery time following infusion of inulin. In the normal subjects, analysis of the cumulative post-infusion recoveries shows that over 95 per cent of the ultimate total recovery was collected at the end of 12 hours, and over 98 per cent of the ultimate recovery was collected at the end of 15 hours (Figure 3). In the patients with edema of congestive failure the quantity of inulin recovered in 22 to 30 hours was 98 per cent of the ultimate total amount recovered in 48 to 72 hours.

Inulin volume of distribution. In the seven

normal subjects the inulin space ranged from 14 to 18 per cent of body weight, with an average of 16.2 per cent. No attempt was made to correlate inulin space with body surface area, but it was noted that higher values in terms of per cent of body weight were obtained in lean individuals. Precision of the measurement may be judged by the reproducibility of values in repeated determinations on the same individual. In the five subjects in whom long infusions (exceeding five hours) were repeated, the largest inulin space difference observed in successive measurements was 0.5 liters and the smallest difference was 0.1 liters.

Inulin space in five patients with congestive heart failure ranged from 18.5 to 39.4 per cent of body weight and correlated directly with the degree of edema, as judged clinically. These values, however, may still be low because of failure to attain equilibrium despite infusion for 26 to 30 hours.

Comparison with other methods. Simultaneous thiocyanate, bromide and Na^{24} spaces were all significantly higher than inulin space (Table I).

DISCUSSION

That inulin does not penetrate cells seems probable *a priori* because of its high molecular weight and elongate configuration (28, 29). It does not enter erythrocytes (30, 31), bile (21, 32), gastric juice (31, 32) or cells of the renal tubule (30, 33), and no enzymes are known to be present in man which are capable of hydrolyzing it (30, 33). It is neither metabolized nor stored in the body as evidenced by its rapid quantitative urinary excretion (Table II). Our six urinary recoveries of inulin are in accord with the observations of others (34-36).

Our present values for the volume of distribution of inulin average 16.2 per cent of body weight as compared with significantly higher values obtained for the volume of distribution of thiocyanate, bromide and Na^{24} . The volume of distribution of mannitol is estimated to be 16 per cent of body weight in man when allowance is made for the metabolism of this compound (20). It appears, therefore, that inulin and mannitol occupy some constant fraction of body water which is smaller than the fraction occupied by any of the electrolytes used for the measurement of extra-

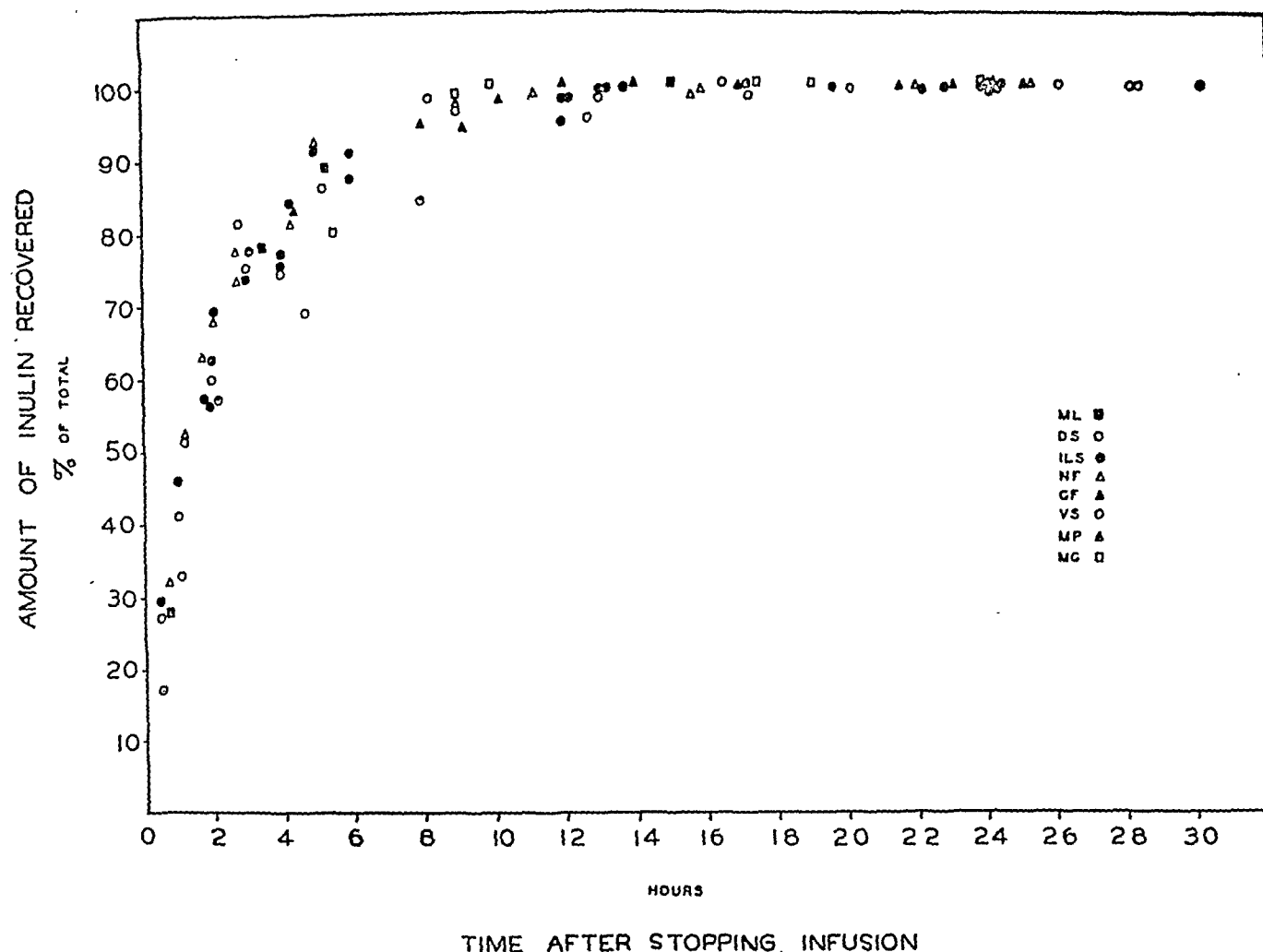


FIG. 3. RATE OF URINARY RECOVERY OF INULIN FOLLOWING CESSATION OF INULIN INFUSION IN EIGHT NORMAL SUBJECTS

cellular fluid (4, 7-10, 12, 14, 37-40). This smaller fraction of the body water conforms better with the values to be predicted from recent total body water measurements in man (41, 42). For these reasons we believe that the volume of distribution of inulin better approximates the true volume of extracellular fluid than the thiocyanate, bromide, chloride or Na^{24} space.

A study of the transcapillary movement of inulin indicates that the time required to establish equilibrium between plasma and interstitial fluid might be reduced if a higher priming dose relative to the sustaining infusion were used. Berger, Farber and Earle (43) have pointed to the importance of adequately estimating the filtration rate, and have shown that only one to one and one-half hours are required to achieve constant plasma inulin levels in normal subjects. Our experience confirms this point. It appears, therefore, that

variations in plasma concentration falling within the analytic error are compatible with incomplete saturation of remote phases of the interstitial fluid.

Extracellular fluid expansion increases the time required to establish plasma-interstitial fluid equilibrium and the time required for recovery of inulin in the urine. This constitutes a serious limitation of the application of the measurement of inulin space in edematous states, especially where there are large accumulations of pleural or peritoneal fluid into which inulin must diffuse. In these cases a similar infusion technique utilizing more rapidly diffusible substances may prove to be more suitable.

Failure of equilibration between plasma and interstitial fluid may result from variation of the filtration rate, the extracellular fluid volume or the infusion rate. For example, in Subject D. S. a pyrogenic reaction accompanied by an elevated

filtration rate was encountered, and the calculated inulin space was higher by 1.4 liters than any other observation of this subject. Infusion of saline over long periods of time may alter the filtration rate or the extracellular fluid volume; therefore, 5 per cent dextrose in distilled water is a preferable diluent for inulin in prolonged infusions.

The presence of inulin in the renal tubules, collecting ducts, pelves and ureters at the moment of stopping the infusion results in an error which raises the calculated inulin volume of distribution. This dead space error varies in the same direction as the renal delay time and inversely with the rate of urine flow at the time of stopping the infusion; however, if urine leaves the renal pelves and ureters in intermittent spurts, an assumed relationship between the renal delay time and rate of urine flow may not afford a reliable correction of the inulin space in this method. The data presented here are not corrected for the renal dead space; but during the infusions in our normal subjects the urine flow was maintained at 2 to 4 cc. per minute in order to minimize this source of error. In patients with congestive failure, where the rate of urine flow is less than 1 cc. per minute, the renal dead space error may increase the calculated inulin space considerably. Direct measurement of the volume and concentration of the urine contained in the renal calyces, pelves and ureters of a dog accounted for 7.6 per cent of a previously determined inulin space. The rate of urine flow in this animal at the time the ureters and renal blood vessels were ligated was 0.8 cc. per minute (31).

The determination of inulin space in patients with edema and oliguria involves both the error from failure to effect equilibrium distribution of inulin and the error of the renal dead space. Although these errors tend to cancel each other to some degree, it is necessary in such cases to prove that plasma and interstitial fluid concentrations are the same by analysis of the edema fluid and to minimize the renal dead space error by instituting diuresis shortly before the infusion is stopped.

SUMMARY

1. In normal human subjects after a priming dose and a constant intravenous infusion of inulin:

- (a) A period of five hours is sufficient for essentially homogenous distribution of inulin throughout the volume of distribution.
- (b) A period of 12 to 15 hours is required for quantitative urinary recovery of the inulin present in the body after the infusion has been discontinued.

2. The volume of distribution of inulin, determined by dividing the total amount recovered in the urine after stopping the infusion by the concentration in plasma water at equilibrium, averages 16.2 per cent of body weight. This value is significantly lower than the volumes of distribution of thiocyanate, bromide and Na^{24} determined simultaneously with the inulin space or in successive experiments.

3. Inulin space is reproducible in the same individual with different durations of infusion in excess of the minimum infusion time required for equilibrium.

4. The calculated inulin volume of distribution in five patients with congestive heart failure ranged from 18.5 to 39.4 per cent of body weight and correlated qualitatively with the observed degree of edema.

5. Extensions and limitations of the constant infusion principle for the determination of extracellular fluid volume are discussed.

ACKNOWLEDGMENTS

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STUDIES OF THE ROLE OF THE LIVER IN HUMAN CARBOHYDRATE METABOLISM BY THE VENOUS CATHETER TECHNIC.

II. PATIENTS WITH DIABETIC KETOSIS, BEFORE AND AFTER THE ADMINISTRATION OF INSULIN^{1,2}

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The recent development of methods for the estimation of the blood flow and metabolism of the liver in man has made it possible to investigate the role of this organ in the normal and deranged metabolic processes of the human being. Previous studies have been concerned with the role of the liver in the carbohydrate metabolism of the normal human being (1). Similar investigations of patients with decompensated diabetic ketosis are reported in the present paper.

METHODS

Hepatic venous blood was obtained by a catheter passed through the right antecubital vein into a right hepatic vein. Blood was drawn from a Cournand needle lying in the femoral artery. The hepatic blood flow was estimated by the bromsulfalein extraction method of Bradley *et al.* (2). The technics of analysis have been discussed previously (1). In addition to studies of glucose and urea, ketone bodies were determined by the method of Weichselbaum and Somogyi (3). Throughout this paper, ketone bodies are expressed as the sum of acetone plus acetoacetic acid plus beta-hydroxybutyric acid (*i.e.*, total ketone bodies). The standard errors of the various technics in our laboratory have been as follows: Glucose, ± 1.0 mg. per 100 ml. at a level of 100 mg./100 ml.; urea, ± 0.1 mg. per 100 ml. at a level of 30 mg./100 ml.; ketone bodies, ± 8.2 mg. per 100 ml. at a level of 100 mg./100 ml.

After several pretreatment observation periods, insulin was injected intravenously. In one case, an infusion was given, at a rate of 1 unit per minute. All other patients received 25 or 50 units every 30 minutes. All insulin used was Lilly "regular" insulin. Samples were collected simultaneously from the femoral artery and the hepatic vein at 15 minute intervals. No glucose was adminis-

tered during the test. All patients had been fasting for at least 18 hours before the beginning of the observations. An accurate voided urine was collected for the 180 minute period of the experiment, and analyzed for glucose and ketone bodies.

CLINICAL MATERIAL

Five patients with diabetes mellitus were studied. These patients had been well controlled on insulin and a diet containing more than 150 grams of carbohydrate prior to the study, but had received no insulin for periods of 36 to 72 hours before the investigation was begun. No disease other than diabetes was evident, and none of the subjects were obese. Urine tests for ketone bodies were positive in all patients at the time the observations were started.

CALCULATIONS

The arterio-hepatic venous difference (A-HV) was obtained by subtracting the hepatic venous concentration from the arterial concentration. A positive value for this difference indicates that the splanchnic system was retaining the substance in question. By multiplying A-HV by the estimated hepatic blood flow (EHBV), the total splanchnic balance could be obtained. The relationship of this value to the hepatic balance is discussed below.

In the case of the ketone bodies, the large standard error of the method made it impossible to determine the value of A-HV with confidence in a single observation. The mean value of A-HV for the entire period was estimated by plotting graphically the arterial and hepatic venous concentrations against time, and integrating the difference with a polar planimeter. It was thus possible to obtain the mean difference of several estimations.

The rate of formation of glucose precursors from protein was estimated from the rate of urea production, assuming that the synthesis of 1 gram of urea releases 1.4 grams of glucose. The justification for this assumption has been discussed previously (1).

The rate of peripheral utilization of glucose and ketone bodies was calculated by subtracting the urinary excretion from the hepatic output. The caloric equivalents of these substances have been estimated by multiplying the value for glucose by 4.0 and for ketone bodies by 5.0 calories per gram. This has been compared with the

¹ Presented at the meeting of the American Society for Clinical Investigation, May 3, 1948, Atlantic City, New Jersey.

² Part of the data presented in this paper have previously been published in abstract form (J. Clin. Invest., 1948, 27, 526).

"normal" basal metabolic requirement obtained from the tables of Aub and DuBois.

RESULTS

Hepatic blood flow: The mean hepatic blood flow appeared to be slightly higher than normal (Table I); however the difference was not significant chiefly because of the wide scatter of the values in the diabetic patients. Inspection of the table, however, indicates that the estimated hepatic blood flow varies directly with the total arterial ketone level. The correlation coefficient of this relationship is 0.86, and the value of p^3 less than 0.01. There was no significant relationship between the hepatic blood flow and the carbon dioxide combining power (Figure 1).

Hepatic glucose output in diabetic ketosis: The mean rate of glucose release by the splanchnic system in this group of patients was 6.9 ± 1.73 mg./kg./min. (Table I). This may be compared

³ P from Fisher's tables. A significant value is considered to be one less than 0.02. All calculations were based on the technics for evaluation of small samples.

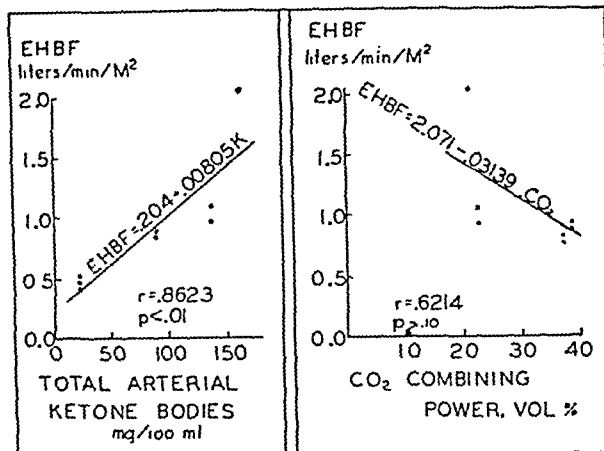


FIG. 1. RELATIONSHIP OF HEPATIC BLOOD FLOW TO ARTERIAL KETONE BODY CONCENTRATION (LEFT) AND CARBON DIOXIDE COMBINING POWER (RIGHT)

with the value of 3.4 ± 0.7 mg./kg./min. in a group of normal individuals (1). The difference is significant (P less than 0.02).

It was possible to calculate the amount of glucose lost in the urine of three of the patients. In Table I it may be seen that in all cases the hepatic

TABLE I
Glucose, urea and ketone body production by the liver in diabetic ketosis

Patient	Age Sex	Area M ² Wt. kg.	EHBF	CO ₂ comb. power	Arterial ketone bodies	Glucose output	Urea output	Glucose from protein	Ketone body output	Normal BMR	Glucose calories	Portion of normal from BMR glucose	Ketone calories	Portion of normal BMR from ketone bodies
			ml./ min./ M ²	vol. per cent	mg./ 100 ml.	mg./ min./ kg.	mg./ min./ kg.	per cent of total glucose	mg./ min./ kg.	cal./ min./ M ²	per min./ M ² †	per cent	per min./ M ² †	per cent
J. L. G.	15 M	1.35 38.5	930 900	38.5		8.1	2.70	42.5		.766	.926	121		
J. P.	21 F	1.53 55.0	2040	21.5	162.0	13.1	2.70	29.2		.616	1.889	306		
W. B.	16 M	1.39 43.4	894 842	37.2	90.6	3.8	0.32	11.8	3.1	.743	.311	42	.479	58
J. W.	51 M	1.72 67.0	956 1095	22.5	137.7	4.6	0.51	8.2	4.0	.623	.479	77	.779	118
J. W. C.	24 M	1.75 61.2	526 430 482		27.1	4.7	0.52	14.1	0.12	.658	.444	67	.016	2
Mean			910			6.9	1.35	21.2			.810			
St. error			145			1.73	0.55	6.4			.289			
Normal mean*			851			3.5	0.21	7.3			.459			
St. error			71			0.70	0.05	1.0			.094			
P			>0.50			<0.02	<0.02	<0.02			>0.10			

* From previously reported normal human subjects (1).

† Total hepatic output, less loss in urine. This is equivalent to peripheral utilization.

output was sufficient to account for the entire urinary glucose loss, and also to supply from 42% to 77% of the normal basal energy requirement.

Urea production in diabetic ketosis: The rate of formation of urea in the five diabetic patients averaged $1.35 \pm .55$ mg./kg./min. as compared with 0.21 ± 0.05 mg./kg./min. in the normal human being (1), a significant difference.

The increased rate of urea formation reflects an increased rate of protein catabolism, which contributes to the glucose produced by the liver. The calculated amount of glucose produced from protein in the diabetics ranged from 8.2% to 42% of the total hepatic glucose output, with a mean of $21.2 \pm 6.4\%$. In normal fasting human beings, the mean conversion percentage was $7.3 \pm 1.0\%$

(1). The difference is significant. No relationship could be established between the rate of protein breakdown and the hepatic blood flow, the arterial ketone body level, the arterial glucose level or the carbon dioxide combining power.

Hepatic ketone body production in diabetic ketosis: The output of ketone bodies by the splanchnic system varied from 0.12 to 4.0 mg./kg./min. After subtracting the urinary ketone body loss, these substances were found to supply a caloric equivalent to the peripheral tissues of from 2% to 118% of the calculated total basal metabolic requirement (Table I).

The effect of insulin on the carbohydrate metabolism in diabetic ketosis: The protocols of the five patients during treatment are shown in Table

TABLE II
Effect of insulin on the splanchnic glucose balance in decompensated diabetic patients

Patient	Pretreatment				15 Minutes				30 Minutes				45 Minutes			
	A	HV	EHBF	Balance	A	HV	EHBF	Balance	A	HV	EHBF	Balance	A	HV	EHBF	Balance
J. L. G.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.
	313	330	1255	-219	248	277	1215	-474	220	245	1340	-335	202	218	1132	-182
	285	331	1255	-576												
	310	322	1215	-148*												
J. P.	436	466	3120	-718†	424	448	1865	-485	390	420	1470	-442†	364	367	1222	-37
W. B.	241	255	1170	-164†	239	242	1292	-40	212	224	1274	-153†	186	202	1200	-192§
J. W.	610	646	1640	-591	572	628	1950	-1092	550	603	2820	-1495†	466	487	2184	-458
	651	630	1640	+163												
	626	652	1885	-490†												
J. W. C.	416	432	1612	-258	408	412	1205	-48	362	357	1178	+59†	310	313	1224	-37
	450	474	1318	-317†												

Patient	60 Minutes				75 Minutes				90 Minutes			
	A	HV	EHBF	Balance	A	HV	EHBF	Balance	A	HV	EHBF	Balance
J. L. G.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.	mg./100 ml.	mg./100 ml.	ml./min.	mg./min.
	196	183	2430	-327	158	165	2430	-170	—	—	—	—
J. P.	342	340	1251	+25	332	329	1251	+38	324	291	1251	+413
W. B.	164	164	1920	0	142	147	1920	-96	141	131	1920	+192
J. W.	483	476	1730	+121†	491	484	2322	+163	402	393	2322	+209
J. W. C.	282	265	1278	+215†	248	243	1467	+74	227	217	1467	+147

* After this observation, a clysis of insulin, 1 unit/minute was given intravenously.
† After this observation, 50 units of insulin were given intravenously.
‡ After this observation, 25 units of insulin were given intravenously.
§ At this time, the patient received 50 ml. of water by mouth.

TABLE III

Arterial, hepatic venous and arterio-venous differences of urea following treatment of diabetic ketosis with insulin (mg./100 ml.)

Patient	Pretreatment			15 Minutes			30 Minutes			45 Minutes			60 Minutes			75 Minutes			90 Minutes		
	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV	A	HV	A-HV
J. L. G.	40.4 30.0 38.0	43.1 38.6 35.4	-2.7 -8.6 -2.6*	33.9	35.8	-1.9	32.9	33.2	-0.3	32.3	31.0	+1.3	30.8	29.1	+1.7	29.3	27.9	+2.4	—	—	—
J. P.	44.8	49.6	-4.8†	45.5	47.6	-2.1	44.8	50.0	-5.2†	44.5	47.2	-2.7	44.6	47.3	-2.7	40.8	45.2	-4.4	44.8	42.8	+2.0
W. B.	34.1	35.2	-1.1†	30.8	32.5	-1.7	28.9	31.6	-2.7†	29.3	31.5	-2.2‡	30.5	30.3	-0.2	29.3	29.7	-0.4	28.9	28.1	+0.8
J. W.	88.6 87.8 86.6	89.4 88.4 88.4	-0.8 -0.6 -1.8†	87.0	88.4	-1.4	86.6	86.2	+0.4†	89.8	87.8	+2.0	87.2	85.4	+1.8†	85.6	84.0	+1.6	81.6	86.5	-4.0
J. W. C.	36.2 37.4	38.2 37.8	-2.0 -0.4†	34.6	37.2	-2.6	34.8	34.6	-0.2†	33.4	33.4	0.0	32.6	33.2	-0.6‡	30.2	31.8	-1.6	29.8	30.8	-1.0

* After this observation, a clysis of insulin, 1 unit/minute, was given intravenously.

† After this observation, 50 units of insulin were given intravenously.

‡ After this observation, 25 units of insulin were given intravenously.

§ At this time, the patient received 50 ml. of water by mouth.

II. Within 15 minutes after the first injection of insulin a decrease in the arterial glucose level occurred. The decline was maintained throughout the entire treatment period, except in the case of J. W., after 45 minutes, and W. B., after 75 minutes. In the latter case, no insulin had been administered for 45 minutes when the arterial concentration of glucose levelled off.

In all cases except one, the splanchnic glucose balance remained negative, *i.e.*, the liver continued to pour out glucose into the circulation, until at least 45 minutes after the beginning of therapy. In the case of J. W. C., a temporary positive balance was established at 30 minutes. This patient had very mild ketosis, shown by a total arterial ketone body level of only 27 mg./100 ml.

In two cases (W. B. and J. W.), there was a temporary increase in the rate of glucose production shortly after the institution of insulin therapy.

The effect of insulin on the ketone body metabolism in diabetic ketosis: The ketone body level in the blood fell during treatment with insulin. The inaccuracies of the analytical method used make it impossible to ascertain the role played by the liver in this decline.

The effect of insulin on the urea metabolism in diabetic ketosis: Before treatment, all patients had negative values for A-HV, indicating that the splanchnic system was adding urea to the blood. In each instance, as treatment progressed, at least one observation was made in which A-HV was

positive (Table III). It was not possible to correlate this occurrence with the status of the glucose balance, although in two patients, the urea and glucose balance patterns were nearly parallel (J. L. G. and J. P.). The significance of this apparent retention of urea by the splanchnic system is discussed below.

DISCUSSION

The hepatic venous catheter technic permits one to estimate the metabolism of the entire splanchnic system. Since it is impossible at present to obtain portal blood from the intact human subject, it is not feasible to separate the activity of the liver from that of the extra-hepatic splanchnic organs. It is possible, however, to predict in some degree the direction and magnitude of the error which is introduced if one assumes that the splanchnic metabolism is equivalent to that of the liver.

It has previously been shown (1) that in the normal human subject, the error introduced by lack of data pertaining to portal glucose concentration is small under basal conditions. Because of the utilization of part of the glucose traversing the digestive organs and the spleen, there is a tendency to minimize the apparent amount of glucose contributed to the circulation by the liver. In the diabetic individual, however, the uptake of glucose by most tissues is reduced (4). This would decrease the magnitude of the error. In diabetic dogs, for example, the portal venous glucose concentration has been observed to be 3 mg./100 ml.

lower than the arterial, at an arterial concentration of 336 mg./100 ml. (5). This small difference could not be observed by the methods used for the present study. For the uncontrolled diabetic patient, therefore, splanchnic and hepatic glucose balances are probably essentially the same.

After increasing the utilization of glucose, by the administration of insulin, the hepatic and splanchnic balances can no longer be considered equivalent. As in the case of the normal subject, however, the error will always tend to minimize the glucose contribution of the liver. No data are available from which the magnitude of this effect can be estimated. The terms "splanchnic" and "hepatic" may therefore be used interchangeably in speaking of the glucose metabolism of the untreated diabetic, but the results of observations by the liver catheter technic after the administration of insulin must be considered only as reflections of the glucose metabolism of the entire splanchnic system.

The extra-hepatic splanchnic organs neither utilize nor produce urea. It is, however, impossible to estimate the amounts of urea which may be withdrawn from the blood and released into the intestinal tract in the bile, gastric, pancreatic and intestinal juices. This loss of urea into the gut may explain the apparent retention of urea by the splanchnic system in the treated diabetic patient. It should be noted, however, that the digestive juices contain urea in a concentration approximating that of the blood (6). The secretion of these juices would not, therefore, be expected to alter the concentration of urea in the blood traversing the splanchnic system.

The apparent splanchnic urea balance might also be altered by dilution of the blood passing through the gut, either by absorption of ingested water or by release of water formed by the oxidation of hydrogen-containing foodstuffs. The first source was eliminated by examining the patients in a post-absorptive state. In one instance, a patient received a drink of water by mistake during the procedure. A definite depression of the hepatic venous urea level occurred in the next specimen (Table III, W. B., 60 minutes).

Water of oxidation is not sufficient to produce a perceptible alteration of hepatic venous concentration. The splanchnic oxidation of 180 mg. of glucose per minute (0.72 calories, equivalent to

about one-half of the basal metabolic requirement) would reduce the hepatic venous urea concentration by only about 0.1 mg./100 ml., a difference within the error of the method of determination.

Although the quantitative relationships between the splanchnic balance and the hepatic balance of the substances studied is somewhat complicated, it should be emphasized that the chief cause of the alterations observed in the splanchnic balance is the activity of the liver.

The large error of the method for determining ketone bodies prevented the estimation of the small A-HV differences directly, and therefore no attempt was made to draw conclusions about the hepatic or splanchnic balances in short observations in the present study. Considerable conservatism should be exercised even with regard to the estimation of A-HV by planimetric methods. The results obtained in this manner are probably of the right order of magnitude, but cannot be considered as reliable as the glucose or urea determinations.

In spite of the small number of observations, a significant direct correlation has been found between the hepatic blood flow and the arterial ketone body level. It is not possible to determine whether the elevated ketone body concentration causes the increased blood flow, or whether both reflect some more basic derangement. This observation is consistent with the findings of Howarth, McMichael and Sharpey-Schafer (7) that in severe diabetic acidosis the cardiac output is normal or increased, and the peripheral vascular resistance is diminished. It is possible that the enhanced hepatic blood flow merely reflects the reduced vascular resistance of the extra-hepatic components of the splanchnic system. In view of the increased liver oxygen requirement for carrying on deamination and ketone body formation, however, it seems probable that the augmented hepatic blood flow is of importance in supplying oxygen needed for the pathologic metabolic activities.

The small number of observations probably accounts for the fact that the correlation between hepatic blood flow and the carbon dioxide combining power was not significant.

Calculation of the glucose balance showed a higher rate of release from the liver of the untreated diabetic patient than the normal human being. This finding is somewhat more definite

than the findings of Crandall and Lipscomb (8) in dogs. These authors found that pancreatectomized dogs released more glucose from their livers than did normal dogs, but the increase was not significant statistically. The apparent difference between the results in humans and dogs may be caused in part by the very high value of EHBV in patient J. P.

It has frequently been shown that the rate of peripheral glucose utilization is increased when the blood glucose level is raised (9). In spite of the high dextrose concentrations found in the diabetic patients, however, carbohydrate utilization was not increased as compared to normal subjects previously studied by the same technic (1). These data, therefore, indicate an increased hepatic glucose output and a relative under-utilization of carbohydrate in the decompensated human diabetic patient. It seems probable that the increased arterial glucose level, made possible in part by the high rate of glucose release by the liver, permits the diabetic tissues to utilize normal quantities of glucose.

It has been shown in diabetic human beings (10) and in animals made diabetic by pancreatectomy (11) or by alloxan (12) that the liver glycogen is usually decreased when the subjects are in severe ketosis. The increased glucose output could not, therefore, be explained merely on the basis of increased glycogenolysis. It can, however, be accounted for almost entirely by increased proteolysis. The finding of increased urea production and protein breakdown by the decompensated diabetic liver agrees with the findings of others by means of balance studies in man (13) and in animals (14). The increased formation of glucose accompanied by an increased rate of urea formation is consistent with Stetten's observations (15) that in alloxan-diabetic rats or rabbits there is a normal or increased rate of glycogen formation from substances smaller than hexose. In our patients, it appears that an increased availability of such glucose precursors occurred as a result of the deamination of amino acids in the liver. The resulting nitrogen-free fragments of amino acids appear to have been incorporated into carbohydrate and to account in large part for the increased glucose production.

Ketone bodies were also found to contribute to the metabolic pattern of the decompensated dia-

betic patient. As might have been anticipated, the percentage of the metabolic needs supplied by ketone bodies was proportional to the degree of ketosis.

The metabolic pattern presented in Table I is expressed in terms of percentage of normal basal metabolic rate supplied by glucose and by ketone bodies. In each case, except that of J. W. C., the mildest case, these percentages add up to more than 100%. This is probably chiefly because patients in diabetic ketosis are not in a basal condition. It should also be emphasized that the fact that a material is being retained by the tissues does not necessarily mean that it is immediately being used for the production of energy. It is not possible by the technics used to estimate the importance of substances previously stored as fat, muscle glycogen, etc., in supplying the metabolic requirements of the organism. Moreover, the actual metabolic rate was not determined in these patients at the time of observation. The percentages presented, therefore, do not reflect the portion of the actual metabolic needs supplied by each of the components.

Presumably, the glucose retention following the injection of insulin reflects the formation of glycogen by the liver, although an enhanced formation of fat in the liver and viscera probably also takes place. The effect of the extra-hepatic viscera, as mentioned above, is to cause an apparent increase in the retention of glucose by the splanchnic system. The delay in the retention of glucose by the splanchnic viscera is therefore shortened by the effect of the activity of the extra-hepatic splanchnic system. The apparent delay of one hour before glucose was retained by the splanchnic system therefore implies that an even longer time was necessary before the liver began to retain glucose. Direct observations of the liver glycogen of diabetic patients (10) have shown that after treatment with insulin the glycogen concentration increases slowly at first and accelerates as therapy proceeds. This is consistent with the observations in the present series of patients.

In two patients, there was actually a considerable increase in the rate of release of glucose by the liver after the administration of insulin. The presence of glycogenolytic substances in commercial insulin has recently been emphasized (16). The

effect noted in these patients may have been due to the presence of this material in the insulin.

After the administration of insulin, there was a decrease in the rate of urea formation until all patients, at one time or another, had a positive splanchnic balance. As discussed above, some part of this retention of urea may have been artifact. In certain instances, however, the retention was of such magnitude that this effect seems insufficient to account for them (Table III, J. L. G., 45, 60, and 75 minutes; J. W., 45, 60, 75 minutes; J. P., 90 minutes). One is therefore tempted to consider the possibility that urea may have re-entered the body economy of these patients. Schoenheimer (17) has shown that in normal fed rats 96% of orally administered nitrogen-labeled urea is recovered unchanged in the urine. Leifer *et al.* (18), however, have shown that up to 20% of the radioactivity of injected carbon-labeled urea may be recovered in the expired carbon dioxide within three hours. Certainly the period of recovery from diabetic acidosis should be one of the best times to see any anabolic activity of urea which is perceptible by ordinary chemical methods of study. The effect of insulin on protein metabolism, in any case, appears to be dramatic. The inhibition of hepatic urea formation occurs so early that it preceded or paralleled the carbohydrate effects in all cases. J. L. G., for example, had a positive splanchnic urea balance after 45 minutes of treatment, but did not begin to retain glucose even after 75 minutes.

SUMMARY

Five patients with uncontrolled diabetes mellitus have been studied by the liver catheterization technic. There was an increased rate of splanchnic glucose and urea production as compared with normal subjects. As the arterial ketone body concentration rose, the hepatic blood flow also increased. There was a normal rate of utilization of glucose in the peripheral tissues. Ketone bodies were also utilized.

After the administration of insulin, the arterial glucose level fell and the rate of splanchnic glucose production decreased. After latent periods of from 45 to more than 75 minutes, the splanchnic system began to retain glucose, presumably chiefly as glycogen. In two instances, there was a considerable temporary increase in hepatic glu-

cose production immediately after the injection of insulin. This may have been due to the effect of the insulin glycogenolytic factor.

The rate of urea production was rapidly reduced by insulin. In all instances, the splanchnic system retained urea for one or more experimental periods while under the influence of insulin. This effect occurred at least as rapidly as the effects on carbohydrate metabolism.

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STUDIES OF THE PULMONARY CIRCULATION. I. THE CIRCULATION TIME FROM THE PULMONARY ARTERY TO THE FEMORAL ARTERY AND THE QUANTITY OF BLOOD IN THE LUNGS IN NORMAL INDIVIDUALS^{1, 2}

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The measurement of the volume of blood contained within the vessels of the lungs has excited considerable interest for many years. A direct method of measurement by clamping the pulmonary vessels, removing the lungs and determining the amount of hemoglobin contained therein has been used on laboratory animals (1). A method that is applicable to intact animals and to human beings was developed by Stewart (2), using the formula $Q = \frac{TV}{60}$, where V represents the minute volume of the heart, Q the volume of the blood in the lungs and T the mean pulmonary circulation time in seconds. By measuring V and T , Stewart found that the average pulmonary blood volume (Q) in a group of dogs amounted to 16 per cent of the total blood volume.

Blumbart and Weiss (3) determined the pulmonary circulation time in normal human subjects using an active deposit of radium. The material was injected into an antecubital vein of one arm and the time of arrival in the right heart and the brachial artery was noted. The term "crude pulmonary circulation time" was used to refer to the difference between the time of arrival of the radium in the right heart on the one hand, and the time of its arrival in the brachial artery on the other. From the "crude pulmonary circulation time" and the cardiac output the pulmonary blood volume was estimated.

The assumption was made both by Stewart (2)

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and by Blumgart and Weiss (4) that the most rapid circulation time does not differ significantly from the mean circulation time. Hamilton, Moore, Kinsman, and Spurling (5) measured the dye concentration in serial samples of blood obtained from the femoral artery immediately after the injection of a dye into a peripheral vein. When the concentration of dye in each sample was plotted against the time of collection of the sample, a characteristic curve was obtained which demonstrated that the mean circulation time differs considerably from the most rapid circulation time. By studies on artificial models they also validated Stewart's method for determining pulmonary blood volume. In addition, they developed a method of estimating the cardiac output from the concentration curve of the dye. By determining the mean circulation time (from a peripheral vein to the femoral artery) and the cardiac output, the volume of blood between the points of introduction and removal of dye was calculated from Stewart's formula.

The rate of peripheral venous flow is variable (3) and introduces considerable inaccuracy in the estimation of pulmonary blood volume by the method of Hamilton *et al.* (5). The direct injection of dye into the pulmonary artery through a catheter obviates this difficulty by eliminating the circulation time from the peripheral vein to the pulmonary artery. Catheterization of the pulmonary artery also permits accurate determination of the cardiac output (6, 7).

METHOD

Thirteen normal fasting male adults between the ages of 30 and 40 were studied. One was eliminated from the series because of the development of marked anxiety during the procedure resulting in an extremely high car-

diac output. The subjects were either physicians or laboratory workers. A No. 8F or No. 9F catheter was introduced into the pulmonary artery by the method of Cournand, and a No. 18 needle with stylet was inserted into the femoral artery (8). The oxygen consumption was measured with a closed circuit spirometer and samples were drawn from the pulmonary artery and femoral artery for the determination of oxygen content by the method of Van Slyke and Neill (9). Immediately thereafter, 5 cc. of a solution of the dye T-1824 containing 300 mg. of dye per 100 cc. were introduced as rapidly as possible through the catheter into the pulmonary artery, the average time required for the injection of the dye being 0.8 seconds. Time intervals were recorded on a direct writing electric kymograph by an assistant. In order to permit more accurate timing of the initial appearance of the dye in the circulation, 1 cc. of the dye was introduced into the catheter before beginning the injection.⁵ Consecutive samples of blood were collected.

For the determination of the dye curve, the serum was separated and 0.1 cc. was diluted with 2 cc. of saline solution. The dye concentration was then determined in the Evelyn photoelectric micro-colorimeter using a con-

⁵ The No. 9F catheter contains a volume of about 1.8 cc. from the femoral artery in small glass tubes which were arranged in series in a wooden rack. The blood spurting from the needle was allowed to flow directly into the sampling tubes. The average time required for collection of a sample was 1.9 seconds, each tube receiving 0.3 to 0.5 cc. of blood. After completion of the sampling from the femoral artery, the dye remaining in the catheter was washed into the circulation and a sample of blood was taken 10 minutes later. The plasma volume was determined from the concentration of dye in the plasma (10), and the total blood volume from the plasma volume and hematocrit reading.

trol sample of serum similarly diluted with saline as a blank.

To determine the mean circulation time, the concentration of dye in each sample was plotted against the time of collection of the sample on linear graph paper. The time of each sample was determined by measuring the interval between the mean time of the injection of the dye and the mean time of the collection of the sample. After the curve was plotted, the area subtended by the primary dye curve was measured with a planimeter. The vertical line that divides this area into two equal parts represents the mean circulation time.

The cardiac output was calculated by the Fick principle (6). The quantity of blood contained in the pulmonary vessels, left heart, aorta and certain larger arteries (Q) is equal to the

$$\frac{\text{cardiac output (cc./min.)} \times \text{mean circulation time (sec.)}}{60}$$

according to the formula of Stewart (2).

RESULTS

The results are given in Table I, and a typical dye curve is illustrated in Figure 1. In all the experiments, the concentration of the dye in the blood obtained from the femoral artery was at or near zero before recirculation began. In those cases in which recirculation began before the primary curve was completed, the curve was extended to the base line for purposes of calculation (Figure 2). The average circulation time in the 12 subjects was 10.2 seconds with a standard deviation of ± 1.6 , and the average duration of the primary curve was 12.3 ± 2.4 seconds.

TABLE I

Subject	Surface area	Oxygen consumed	Arterio-venous oxygen difference	Cardiac output	Cardiac index	Mean circulation time	Duration of curve	Q	Total blood volume
	sq. m.	cc./min.	vol. per cent	L./min.	L./min./sq. m.	sec.	sec.	cc.	cc.
B. G.	1.78	233	4.01	5.81	3.26	10.5	13.5	1010	5780
A. F.	1.79	193	4.91	3.93	2.19	12.1	12.8	790	5320
J. W.	1.80	272	3.58	7.60	4.20	11.8	14.8	1490	5800
A. L. F.	1.83	239	3.17	7.54	4.12	9.5	11.2	1190	5710
D. N.	1.86	287	3.80	7.55	4.05	7.7	11.0	970	5000
R. W.	1.89	215	4.15	5.18	2.74	10.4	15.0	900	5050
D. B.	1.92	266	3.25	8.18	4.26	12.1	15.8	1650	5760
C. C.	1.93	235	4.15	5.66	2.94	11.4	11.0	1080	6200
F. W.	1.95	304	3.37	9.02	4.61	7.6	8.5	1140	5980
L. Z.	2.07	311	4.39	7.08	3.42	9.4	11.0	1110	6690
H. F.	2.08	292	3.63	8.04	3.86	9.2	8.7	1230	6250
G. P.	2.14	282	3.79	7.44	3.48	10.9	14.0	1350	7860
J. H.*	1.80	251	2.58	9.74	5.41	7.6	5.1	1230	6400
Mean	1.92	261	3.85	6.92	3.59	10.2	12.3	1160	5950
Standard deviation	± 0.38	± 0.37	± 0.50	± 1.46	± 0.72	± 1.6	± 2.4	± 246	± 776

* Omitted in the calculation of mean values because the subject showed evidence of marked anxiety during the procedure.

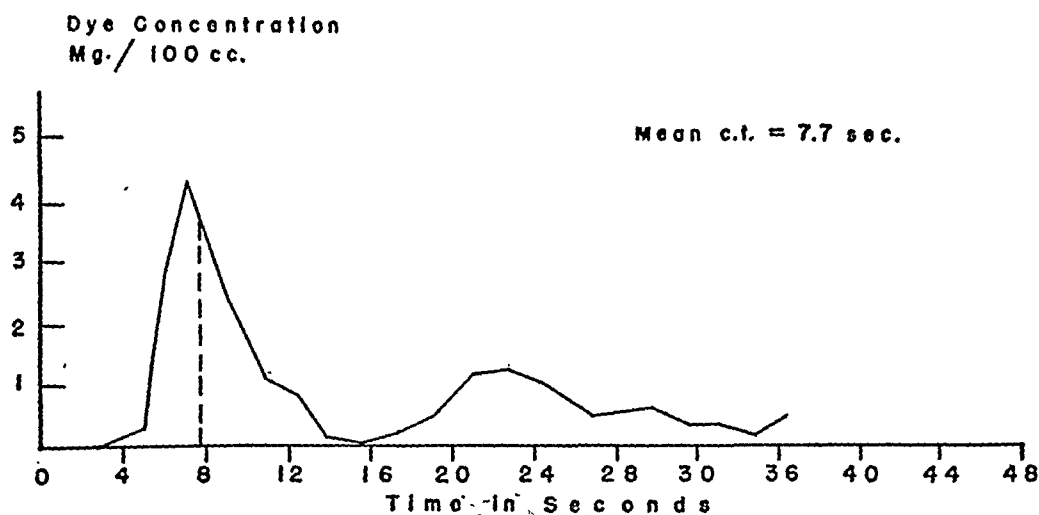


FIG. 1. DYE CONCENTRATION CURVE IN NORMAL SUBJECT

The dye concentration reached zero before recirculation began. The mean circulation time is represented by the broken vertical line.

The cardiac output for the group was 6.92 ± 1.5 , and the cardiac index was 3.59 ± 0.72 liters per minute. The mean value for Q , calculated from Stewart's formula, was 1160 ± 246 cc. Correcting the individual values for Q to 1.73 sq. m. of body surface, gave a mean value for the group of 1045 ± 200 cc. Expressed as per cent of total blood volume, the mean for the group was 19.5.

DISCUSSION

As emphasized by Hamilton (5), the mean circulation time differs from the most rapid circulation time. The primary dye curve from which the mean circulation time is calculated is produced by dye particles travelling from the pul-

monary artery to the femoral artery at different rates of speed. In the study by Blumgart and Weiss (4), the most rapid circulation time rather than the mean circulation time was determined. If the apparatus for detecting the arrival of the radium in the brachial artery were extremely sensitive, the most rapid circulation time would differ considerably from the mean circulation time. If, however, the time recorded really represents the peak concentration of the substance, the value should correspond closely to the mean circulation time. As can be seen in Figure 1, the mean circulation time differs little from the time required to attain the maximum concentration of dye in the normal subject. It is of interest that the circula-

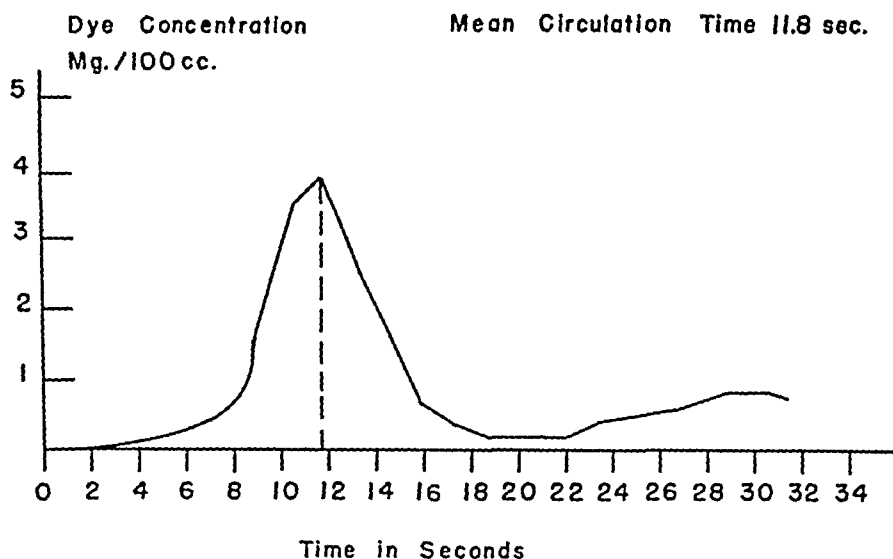


FIG. 2. DYE CONCENTRATION CURVE IN NORMAL SUBJECT

The dye concentration did not reach zero before recirculation began. The mean circulation time is represented by the broken vertical line.

tion time from the right auricle to the brachial artery, as measured by Blumgart and Weiss (3), is quite similar to the circulation time from the pulmonary artery to the femoral artery, as measured by the dye method, the average value for the former being 10.8 seconds and for the latter 10.2 seconds.

The value for Q , calculated from the formula of Stewart, represents the amount of blood contained within the pulmonary vessels, left heart, and certain of the larger arteries. Hamilton (5) has pointed out that on the systemic side of the circulation, Q includes the blood in the aorta and iliac arteries together with all the blood contained within the other branches of the aorta to points where dye arrives no later than it does at the femoral artery. It is apparent that in the pulmonary vessels, Q includes only blood which is circulating.

In comparing the value of Q in the present study with that obtained by Hamilton, it should be pointed out that in his experiments, the dye was injected into an arm vein. Thus, the mean circulation time included the time required for the dye to travel from the arm to the pulmonary artery in addition to the time of passage from the pulmonary artery to the femoral artery. Blumgart and Weiss (3) found that the circulation time from the antecubital space of the arm to the right heart was 6.7 seconds. This fact explains the considerably higher value for Q obtained by Hamilton and colleagues in normal subjects (5). The value for Q in their studies was 2 liters as compared with 1.16 liters in the present experiment.

In order to determine the true pulmonary blood volume, it is necessary to know the mean circulation time from the pulmonary artery to the left auricle, a value which was not determined in the present experiments. From the studies available in the literature it is difficult to estimate the true pulmonary circulation time. Nevertheless, it is apparent that the true pulmonary blood volume is less than the value for Q . A more accurate estimation of the true pulmonary blood volume must await a method for determining the mean circulation time from the pulmonary artery to the left auricle.

SUMMARY

1. The mean circulation time from the pulmonary artery to the femoral artery was determined by injecting the dye T-1824 into the pulmonary

artery and measuring the concentration of the dye in consecutive samples of blood obtained from the femoral artery. The mean circulation time in 12 normal subjects was 10.2 ± 1.6 seconds.

2. The volume of blood contained in the pulmonary vessels, left heart, aorta, and certain of the larger systemic arteries was calculated from Stewart's formula using the mean circulation time and the cardiac output as measured by the direct Fick method. The mean volume for the group of 12 normal subjects was 1160 ± 246 cc., or 19.5 per cent of the total blood volume.

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STUDIES OF THE PULMONARY CIRCULATION. II. THE CIRCULATION TIME FROM THE PULMONARY ARTERY TO THE FEMORAL ARTERY AND THE QUANTITY OF BLOOD IN THE LUNGS IN PATIENTS WITH MITRAL STENOSIS AND IN PATIENTS WITH LEFT VENTRICULAR FAILURE^{1, 2}

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The pathogenesis of dyspnea in left ventricular failure and in mitral stenosis is not entirely understood but it is generally conceded that pulmonary vascular congestion is involved (1). Preceding the congestion there is probably an elevation in the left auricular and pulmonary venous pressures but to date it has not been possible to measure such pressures in the human being. Accurate quantitation of the pulmonary vascular congestion itself is difficult and the use of the vital capacity for this purpose is far from satisfactory. Much more promising in this connection is a method, described in a previous paper (2), which measures the volume of blood in the lungs, left heart, aorta, and certain systemic arteries. The method is based on the measurement of the mean circulation time from the pulmonary artery to the femoral artery, and the simultaneous determination of the cardiac output. An increased volume of blood in the pulmonary vessels should result in a corresponding increase in the total volume of blood (Q) measured by the technique. The results of studies based on this premise are presented in the following account.

MATERIAL AND METHODS

A group of 10 men with rheumatic heart disease and mitral stenosis were studied. All these patients had dyspnea on exertion and some degree of pulmonary hypertension. As examples of left ventricular failure, 18

men with disease of the aortic valve or hypertensive heart disease were chosen. All had dyspnea on exertion and had suffered from several episodes of paroxysmal dyspnea in the past, but none were severely orthopneic at the time the study was performed. Two of the patients with rheumatic heart disease and two with left ventricular failure previously had evidence of right ventricular failure manifested by hepatomegaly, peripheral edema and an elevated systemic venous pressure.

A catheter was introduced into the pulmonary artery by the method of Cournand and co-workers (3). The mean circulation time from the pulmonary artery to the femoral artery was measured and the cardiac output was determined as previously described (2). The volume of blood in the pulmonary vessels, left heart, aorta, and iliac arteries, together with all the blood contained within the other branches of the aorta to points where dye arrives no later than it does at the femoral artery (Q) was determined from Stewart's formula (4). The pressures in the pulmonary artery, right ventricle, and right auricle were measured with a Hamilton manometer. The level of the right auricle was determined by the method of Lyons, Kennedy, and Burwell (5). The pulmonary arterial diastolic pressure was measured immediately before auricular systole to avoid artifacts which occur in the pulmonary arterial tracing during auricular systole. The systolic pressure was determined from the right ventricular tracing, since artifacts due to motion of the catheter interfered with this measurement in the pulmonary arterial tracings.

Heart volumes were determined from the teleroentgenogram by the method of Keys *et al.* (6). The predicted normal frontal area of the heart for the individual was estimated from the nomogram of Ungerleider and Gubner (7). The predicted heart volume was obtained by the use of the tables of Keys *et al.* (6).

The vital capacity was measured in the sitting position with a spirometer. The predicted normal figure for the individual was obtained by applying the formula of Baldwin, Cournand, and Richards (8). Four and four-tenths per cent were added to the value obtained by this formula to correct for the difference between the supine and sitting position (9).

¹ Published with permission of the Chief Medical Director, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the authors.

² Presented in part at a meeting of The American Society for Clinical Investigation in Atlantic City, N. J., May 3, 1948.

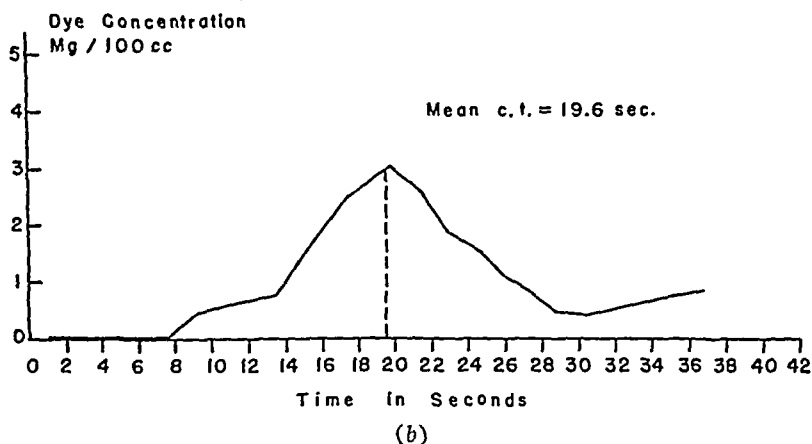
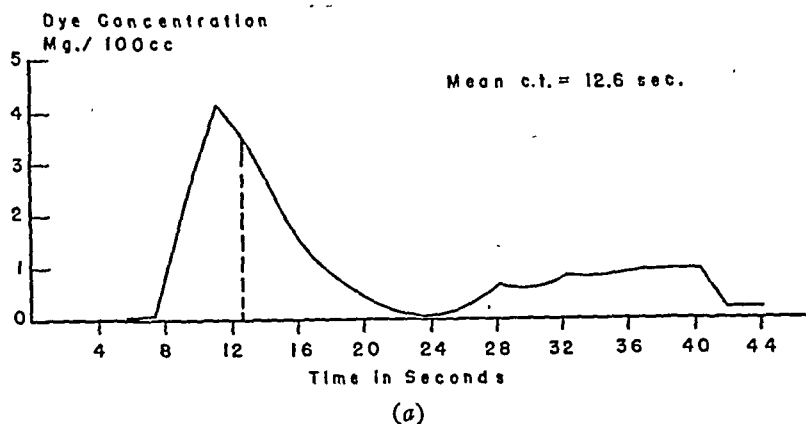


FIG. 1. DYE CONCENTRATION CURVES IN PATIENTS WITH HEART DISEASE

(a) The chart above is the dye concentration curve in a patient with mitral stenosis (Subject R. C.), and (b) the chart below is the dye concentration curve in a patient with left ventricular failure (Subject J. K.).

TABLE I

Cardiac output, mean circulation time and Q in a group of patients with rheumatic heart disease and mitral stenosis

Subject	Surface area	Oxygen consumed	Arterio-venous oxygen diff.	Cardiac output	Cardiac index	Mean circulation time	Duration of curve	Q	Total blood volume	Vital capacity	Pulm. arter. press. systolic	Pulm. arter. press. diastolic	Right ventric. press. diastolic
	sq. m.	cc./min.	vol. per cent	L./min.	L./min./sq. m.	sec.	sec.	cc.	cc.	per cent normal	mm. Hg.	mm. Hg.	mm. Hg.
J. K.	1.58	220	3.36	6.55	4.15	9.2	14.2	1000	5340	95.5	31.9	15.6	1.7
H. H.	1.69	245	8.43	2.91	1.72	24.1	26.0	1170	7726	57.2	83.5	38.5	5.9
M. O.	1.70	253	8.75	2.89	1.70	33.4	45.2	1610	—	60.4	83.5	36.2	13.8
V. S.	1.74	206	4.13	4.99	2.87	13.6	17.4	1130	5060	102.8	33.6	16.5	6.9
M. M.	1.77	284	8.98	3.16	1.79	21.7	32.7	1140	—	47.8	78.1	46.0	1.4
E. M.	1.77	286	5.79	4.94	2.79	15.7	22.0	1290	7710	86.8	43.0	20.3	6.1
F. M.	1.77	272	5.92	4.59	2.59	19.6	26.1	1500	5000	50.4	113.5	61.7	8.6
L. N.	1.79	242	5.30	4.57	2.55	14.0	13.2	1070	7200	52.9	64.0	43.0	7.3
R. C.	1.84	249	4.72	5.28	2.87	12.6	17.8	1110	6510	79.3	68.0	31.0	1.0
G. W.	1.93	270	4.82	5.60	2.90	13.5	20.0	1260	6930	89.3	40.4	22.3	2.8
Mean	1.76	253	6.02	4.55	2.59	17.7	23.5	1230	—	71.2	64.0	33.1	5.6
Standard deviation	±0.10	±26	±2.01	±1.22	±0.74	±7.1	±9.7	±190	—	±19.0	±27.6	±14.9	±3.9

TABLE II
Cardiac output, mean circulation time and Q in a group of patients with left ventricular failure

Subject	Diagnosis*	Surface area sq. m.	Oxygen consumed cc./min.	Arterio-venous oxygen diff. vol. per cent	Cardiac output L./min.	Cardiac index L./min./sq. m.	Mean circulation time sec.	Duration of curve sec.	Q cc.	Heart volume cc.	Total blood volume cc.	Vital capacity per cent of normal	Pulm. arter. press. diastolic mm. Hg.	Pulm. arter. press. systolic mm. Hg.	Right ventric. press. diastolic mm. Hg.
A. D.	R.H.D.—A.I.	1.58	218	5.39	4.04	2.56	32.6	19.3	2200	1440	5500	91.8	12.9	32.0	1.4
J. K.	H.H.D.	1.62	235	6.01	3.91	2.41	19.6	22.4	1280	1023	4730	59.8	12.9	39.0	1.4
R. D.	H.H.D.	1.68	246	7.20	3.42	2.04	27.0	40.1	1540	1491	7810	70.4	24.6	62.3	7.7
B. D.	H.H.D.	1.68	277	6.22	4.45	2.65	18.4	28.4	1360	1202	5200	68.3	30.7	56.5	1.4
H. H.	R.H.D.—A.I.	1.71	295	10.21	2.89	1.70	26.4	41.3	1270	1298	6580	85.0	34.0	73.0	10.8
E. O.	R.H.D.—A.S.	1.71	308	7.16	4.30	2.51	21.0	28.6	1510	1679	6700	73.1	19.4	31.3	2.2
M. S.	R.H.D.—A.S.	1.80	307	4.37	7.03	3.91	14.6	14.6	1710	845	4450	—	18.9	30.6	6.6
E. S.	S.H.D.—A.I.	1.80	264	5.69	4.64	2.58	23.2	24.6	1790	1211	7320	57.0	5.1	20.0	2.3
R. Q.	H.H.D.	1.81	288	5.22	5.52	3.05	24.8	48.8	2280	1440	8100	79.5	13.4	28.6	0.6
L. G.	H.H.D.	1.82	290	5.81	4.99	2.74	22.2	33.5	1850	1144	7080	86.7	23.1	56.7	1.9
E. T.	R.H.D.—A.I.	1.82	279	5.58	5.00	2.75	18.0	31.8	1500	1116	6170	54.6	21.9	29.9	9.0
H. G.	R.H.D.—A.S.	1.84	223	4.63	4.82	2.62	24.4	20.8	1960	1289	6800	73.8	29.0	40.0	1.8
C. M.	S.H.D.—A.I.	1.85	279	4.71	5.92	3.20	17.2	35.9	1700	1260	—	67.8	25.0	41.9	5.8
G. R.	H.H.D.	1.93	293	4.50	6.51	3.37	15.2	24.3	1650	1163	7030	88.8	17.5	40.0	4.2
H. B.	R.H.D.—A.S.	1.93	254	3.68	6.90	3.58	12.0	16.2	1380	915	5550	62.7	12.5	29.2	4.9
C. B.	H.H.D.	1.94	300	4.61	6.51	3.36	22.0	15.0	2390	1192	7490	116.3	15.1	29.3	5.4
A. B.	H.H.D.	2.04	219	5.87	3.73	1.83	21.6	22.8	1340	854	6530	84.4	8.8	22.3	5.1
M. K.	R.H.D.—A.I.	2.06	336	5.04	6.67	3.24	24.8	31.4	2760	1953	—	97.1	14.7	26.3	8.5
	Mean	1.81	273	5.66	5.07	2.78	21.4	27.8	1750	1251	6440	77.5	18.9	38.3	4.5
	Standard deviation	±0.13	±34	±0.39	±1.28	±0.59	±5.0	±9.6	±420	±281	±1090	±16.1	±7.8	±14.7	±3.1

* H.H.D.—Hypertensive Heart Disease.
 R.H.D.—Rheumatic Heart Disease.
 S.H.D.—Syphilitic Heart Disease.
 A.S.—Aortic Stenosis.
 A.I.—Aortic Insufficiency.

RESULTS

In order to determine the significance of the findings in the patients with heart disease it was necessary to compare them with the findings previously obtained in a group of 12 normal subjects (2). The dye concentration curves in patients with heart disease differed in most instances from the normal curve in that they were more prolonged than the normal and the maximal dye concentration was lower in the former than in the latter (Figure 1). Because recirculation usually began before the completion of the primary curve, a small error may have occurred in the determination of the mean circulation time. The mean circulation time was prolonged in the group with mitral disease and in the group with left ventricular failure, the increase being greater in the latter group (Table I). The mean cardiac index was significantly reduced in both groups as compared with the normal subjects.

The mean value for Q in 10 patients with mitral stenosis was 1230 cc. as compared with 1160 cc. in the group of 12 normal subjects ([2] Table I). This difference is not significant statistically. Adjusting the individual values to 1.73 sq. m. body surface resulted in a larger difference between the two means but the statistical significance of the difference remained doubtful.

The mean value for Q in the group of 18 patients with left ventricular failure was 1750 cc. which is

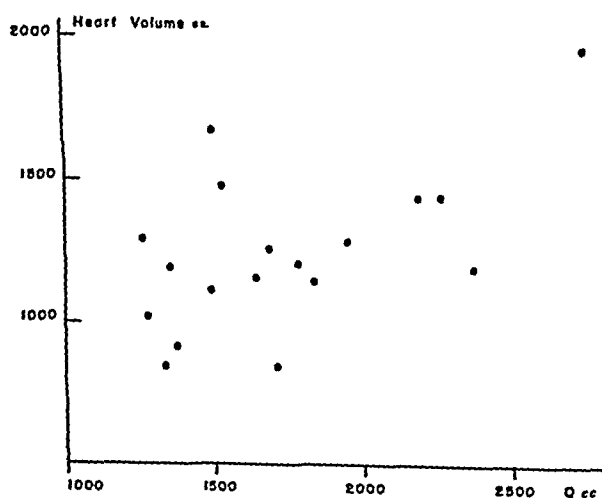


FIG. 2. CHART SHOWING CORRELATION BETWEEN HEART VOLUME AND Q IN PATIENTS WITH LEFT VENTRICULAR FAILURE

TABLE III

Comparison of mean increase in Q with mean increase in volume of the heart in 18 patients with left ventricular failure

	Left ventricular failure	Predicted normal	Mean increase
Volume of heart (cc.) Estimated from teleroentgenogram	1251	675	576
Q (cc.)	1750	1160	590

significantly greater than the mean value for Q in normal subjects (Table II). It was immediately apparent that the increase in Q in the group with left ventricular failure could be due to an increase in volume of blood in the left ventricle. In order to evaluate this possibility, the volume of the heart in the individual cases was plotted against Q (Figure 2). There was a moderate correlation ($r = 0.6954$ $p < .01$). In Table III the difference between the mean volume of the heart in the group with left ventricular failure and the predicted mean normal heart volume for the group is compared with the difference between the mean value for Q in the group with left ventricular failure and the mean value for Q in the group of normal subjects. The two values were very similar.

The pulmonary arterial pressure was moderately elevated in the group with left ventricular failure and more strikingly elevated in the group with mitral stenosis (Tables I and II). There was no correlation between Q and the vital capacity in either group.

DISCUSSION

The prolongation of the circulation time from a peripheral vein to an artery in heart disease is well known and is widely used in the clinical assay of congestive heart failure. In most studies of the circulation time, a method is used which gives a single end point. The means of detecting the end point differs, both in technique and in sensitivity, from method to method. For example, in the determination of the circulation time by the use of radium as performed by Blumgart and Weiss (10), the end point depends on the sensitivity of the detecting device and the concentration of the radium in the blood flowing through the brachial artery. Hamilton *et al.* (11) have demonstrated that this end point does not represent the mean

circulation time but merely approximates the earliest circulation time. A study of the dye concentration curve determined in patients with heart disease, makes it apparent that the measurement of the circulation time by the usual method will give a value which is quite remote from the true mean circulation time.

The importance of the determination of the mean circulation time lies in the fact that it is a function of the rate of blood flow and the volume of blood contained in a given portion of the circulation. According to Stewart (4) and Hamilton (11), the mean circulation time from the pulmonary artery to the left auricle should be inversely proportional to the cardiac output and directly proportional to the volume of blood in the pulmonary vessels. Unfortunately, it is impossible to measure the circulation time from the pulmonary artery to the left auricle so that this approach to the estimation of the pulmonary blood volume is closed. It is possible, however, to measure the mean circulation time from the pulmonary artery to the femoral artery. Using Stewart's formula, one finds that this mean circulation time is inversely proportional to the cardiac output and directly proportional to the value Q , which represents the amount of circulating blood in the lungs, left heart, aorta, and certain other arteries as defined above. Hence, if the cardiac output and the mean circulation time are known, Q can be calculated. If the volume of blood in the aorta and left heart remains constant, variations in the pulmonary blood volume will cause corresponding variations in Q . Blood in the lungs which is stagnant or circulating very slowly would not be measured by this method.

There was no significant increase in Q in the group of patients with mitral stenosis and pulmonary hypertension as compared with the group of normal subjects, and it can be concluded, therefore, that there was no large increase in pulmonary blood volume. Moreover, a marked increase in pulmonary arterial pressure can occur in mitral stenosis without an increase in Q and, consequently, without a large increase in pulmonary blood volume. None of the patients studied had severe orthopnea or evidence of pulmonary congestion and edema roentgenologically. It is quite possible that a marked increase in pulmonary

blood volume does occur in patients with these findings.

There was a marked increase in Q in the group of patients with left ventricular failure. Gernandt and Nylin (12) have observed a relationship between increase in circulation time from peripheral vein to peripheral artery and increase in size of the heart. All patients with left ventricular failure in this study had varying degrees of left ventricular enlargement, and the largest increase in Q occurred in those patients with the largest hearts. A positive correlation existed between the heart volume and Q . If the mean increase in heart volume is compared with the mean increase in Q , the similarity of the two values suggests that they have a common cause, namely, an increase in the amount of blood in the left ventricle. In roentgenological studies of heart volume, it is impossible to distinguish between the effects of hypertrophy and dilatation but it is generally conceded that the increase in the size of the heart in heart failure is primarily due to cardiac dilatation (13, 14). Because of the contribution of the blood in the dilated left ventricle to Q , it is difficult to evaluate changes in pulmonary blood volume in patients with left ventricular failure.

The reduction in vital capacity in patients with heart disease has been attributed to pulmonary congestion with consequent changes in pulmonary distensibility and elasticity (15, 16). It has been suggested that prolongation of the circulation time in association with reduced vital capacity is due to an increase in the volume of blood in the lungs (17). From the present study, it does not appear that reduction of vital capacity is of necessity accompanied by a large increase in pulmonary blood volume, nor does it appear that an increase in pulmonary blood volume is the major factor in prolonging the circulation time.

SUMMARY AND CONCLUSIONS

1. The mean circulation time from the pulmonary artery to the femoral artery and cardiac output were determined on 10 patients with mitral stenosis associated with pulmonary hypertension and 18 patients with left ventricular failure.
2. From the mean circulation time and the cardiac output the volume of blood in the pulmo-

nary vessels, left heart, aorta and certain other arteries (Q) was calculated.

3. The mean circulation time was prolonged and the cardiac output was reduced in both groups as compared with normal subjects. The value for Q in the group of patients with mitral stenosis and pulmonary hypertension did not differ significantly from the value in normal subjects and hence, there was no evidence of a large increase in circulating blood in the lungs.

4. A marked increase in Q was present in the group of patients with left ventricular failure. Because of evidence which suggested that the increase in Q was largely due to an increase in the amount of blood in the left ventricle, it was impossible to evaluate changes in pulmonary blood volume in this group.

5. There was no correlation between the reduction in vital capacity and Q . This suggests that the reduction in vital capacity in patients with heart disease is not a direct function of increase in pulmonary blood volume.

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MATURATION OF RENAL FUNCTION IN CHILDHOOD:^{1, 2} CLEARANCE STUDIES

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Evidence has been presented by several investigators that functional capacity of the kidney in the premature and young infant is lower than in older children and adults. Schoenthal (1) using urea clearance, McCance and Young (2) urea and inulin clearances, Barnett (3) inulin clearance, and Gordon, Harrison and McNamara (4) urea clearance, showed that the infant had a lower glomerular function when compared to the adult on the basis of a unit of surface area. Recently West *et al.* (5) reported a study of renal function in infants during the first two years of life and Barnett *et al.* (6), a study in premature infants. This present study was begun in 1945 to determine the rate of maturation of certain of the kidney functions from the newborn period through childhood. The following functions were estimated: glomerular filtration rate, maximal tubular excretory capacity for para-amino-hippurate, urea clearance, and effective renal plasma flow.³ Sixty-three normal well children between the ages of two days and 12 years were studied.

METHODS

G.F. was determined as mannitol clearance by the single injection technique. A 20 minute period was allowed for

¹ Presented before the Society for Pediatric Research, Stockbridge, Mass., May 1947, and exhibited at the Fifth International Congress of Pediatrics, New York City, July 1947.

² The mannitol and para-amino-hippurate used in these studies were kindly furnished to us through the courtesy of Sharp & Dohme, Inc., Glenolden, Pennsylvania.

³ These abbreviations are used throughout the paper:

G.F. = glomerular filtration rate

P.F. = effective renal plasma flow

Tm_{PAH} = maximal tubular excretory capacity for para-amino-hippurate

F.F. = filtration fraction

PAH = para-amino-hippurate

equilibration before the first specimen was collected. The blood levels were plotted against time on semi-logarithmic paper; the blood level at the midpoint of each urine collection period, minus two minutes, read off the resulting curve. The mannitol levels in the blood when so plotted always formed a straight sloping line (a minimum of three and in the majority of instances four levels were determined). There was no evidence of lack of equilibration of mannitol in the body fluids after the period allowed. P.F. was determined simultaneously as the clearance of sodium para-amino-hippurate (PAH) at low plasma levels (between 0.5 and 3 mg. per 100 cc. of plasma); the tubular excretory capacity was determined by the excretion of PAH at high blood levels (between 50 and 100 mg. per 100 cc. of plasma). In several instances the Tm_{PAH} was calculated using the values for glomerular filtration rate determined in previous periods. In 27 instances where the Tm_{PAH} was determined simultaneously with the last period of mannitol clearance, the value for glomerular filtration rate was not influenced by the high concentration of plasma PAH. The blood level of PAH was maintained by a priming injection and a continuous intravenous infusion. The priming solution of PAH for Tm determination was always diluted in the syringe to three to four times its volume, either with the dilute solution remaining in the infusion tubing or with saline, and injected as slowly as possible. In larger children, where the volume might have been too large for a syringe, the priming solution was allowed to run in through the infusion tubing from an open burette flask. When injected rapidly and in concentrated form, this amount of PAH solution may cause a sensation of intense heat, involuntary defecation, nausea, vomiting, abdominal pain or pain in the extremity used for injection. With the technique just described, these reactions were usually mild or absent; with one exception they ceased shortly after completion of the priming injection. They seemed to be milder in infants than in older children. The test was started in the morning, after the child had had a light breakfast or, in the case of infants, a bottle one to three hours previously and was well hydrated.

The laboratory determinations of mannitol and PAH were carried out according to the methods outlined by Smith *et al.* (7, 8). Two modifications were used: (1) The deproteinization was performed by the Somogyi

method (one part plasma or, for mannitol, two parts yeasted plasma dilution, 10 parts distilled water, two parts 0.3 N barium hydroxide solution, two parts 5% zinc sulfate solution). (2) For the determination of mannitol the mixtures of plasma filtrate and acid periodate solution were left at room temperature overnight instead of being heated in a boiling water bath for 20 minutes prior to titration.⁴

The standard error of the mannitol clearance determination in a combined series of 75 tests with four periods each plus 60 tests with three periods each was 5.7 cc. with a mean error of 4.73 cc. \pm 0.49. The standard error of 103 determinations of renal plasma flow by PAH clearance with three periods each was 55.2 cc./min. with a mean error of 39.75 cc. \pm 6.44. The standard error of 112 determinations of the tubular excretory capacity for PAH, with three periods in each test, was 5.85 mg./min. with a mean error of 3.75 mg. \pm 0.55. These errors include both the laboratory determination and the bedside technique, such as urine collection, timing of specimens, etc.

The urea clearance was determined simultaneously with the mannitol clearance for three periods in each test.

⁴ Note: Incidentally this modification, which in our hands gave more constant results than the original method, may account for the fact that there is no evidence in our figures of the interference of high levels of PAH with the mannitol determination. Other investigators have recently reported such an interference (9).

Urea was determined in blood and urine with urease by the aeration method of Van Slyke and Cullen (10), but with boric acid substituted for the standard mineral acid in the receiving flask. Toward the end of this study, Conway's micro-method with urease, as modified by Steinitz (11), was substituted for the Van Slyke and Cullen method. Both methods give identical results.

It must be made clear that these tests were performed with a relatively high urine flow, resulting from the diuresis produced by mannitol and the intravenous infusion. This high urine flow does not alter the clearance rates greatly, with the exception of the urea clearance which may be somewhat higher than under ordinary conditions. Most of the tests were performed on quiet or sleeping children and, therefore, were presumably not unduly influenced by circulatory changes. Prolonged periods of starvation before performing the test were not deemed necessary. We found that the children were much quieter during the test if they were not hungry.

RESULTS

In Figure 1A we have graphically presented the glomerular filtration rates obtained at different ages throughout the first year. In Figure 1B the glomerular filtration rates of the children over one year of age are recorded in a similar fashion. Table I, column 11, gives the actual values for

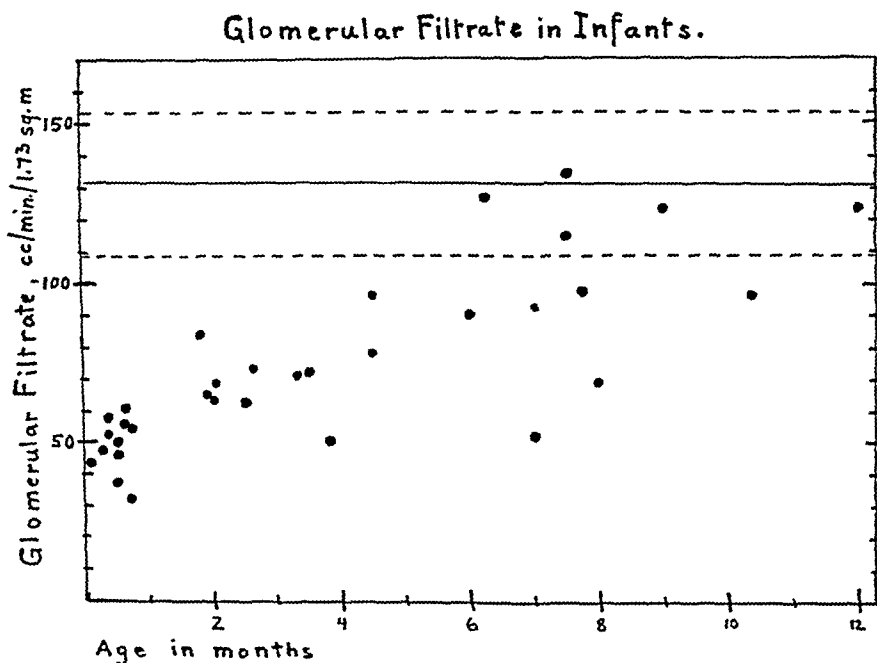


FIG. 1A. GLOMERULAR FILTRATION RATE IN INFANTS

Each dot represents the average value in an individual child. The horizontal line represents the mean adult value of the glomerular filtration rate of an adult with a surface area of 1.73 sq.m. The broken lines represent one standard deviation from this mean, as determined in adults (12).

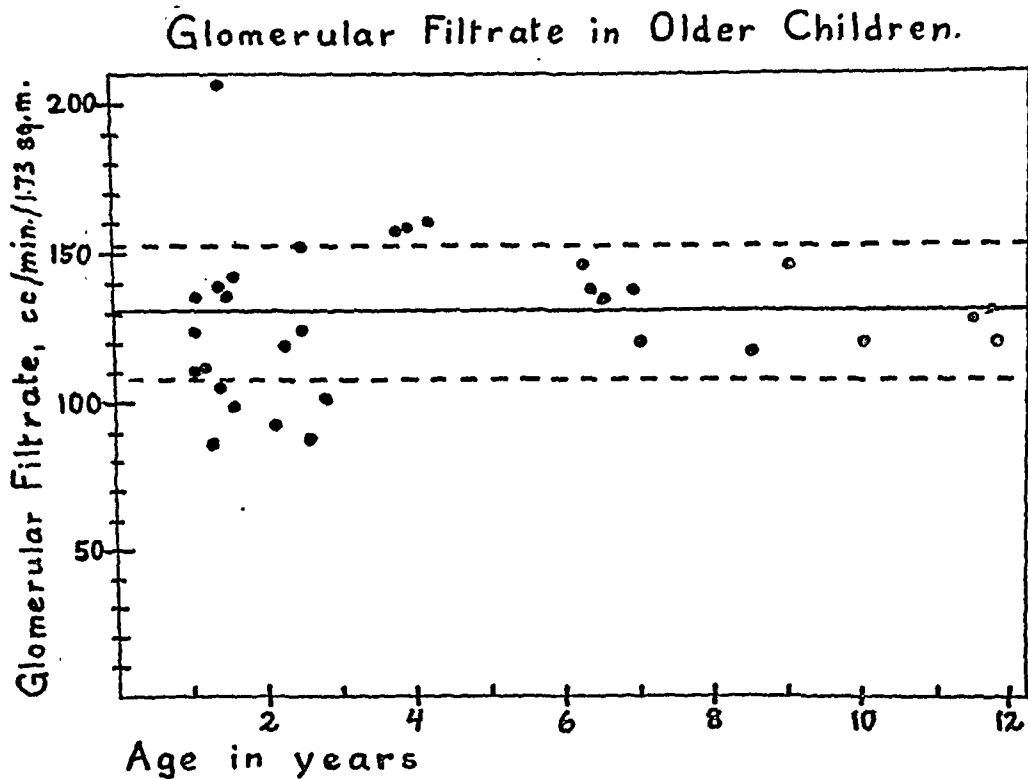


FIG. 1B. GLOMERULAR FILTRATION RATE IN OLDER CHILDREN
Symbols as in Figure 1A.

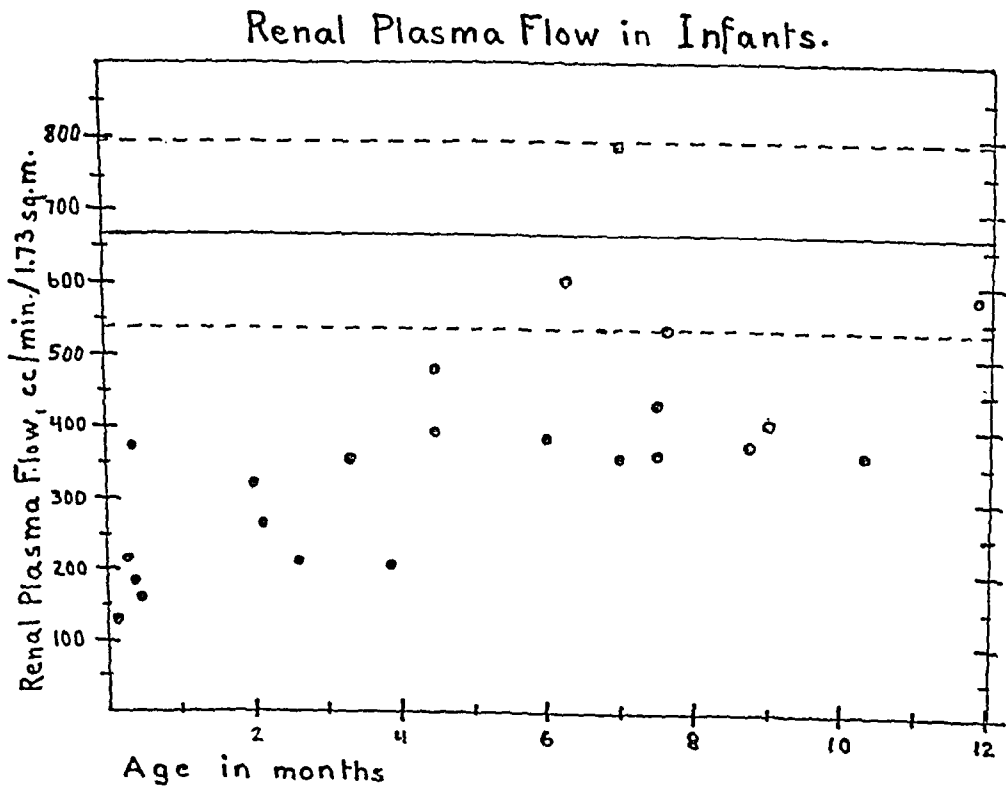


FIG. 2A. EFFECTIVE RENAL PLASMA FLOW IN INFANTS
Symbols as in Figure 1A.

MATURATION OF RENAL FUNCTION IN CHILDHOOD

Renal functions in children, absolute and corrected for surface area, according to age (days)									
8	9	10	11	12	13	14	15	16	

[illegible]

TABLE 1A—Continued

Patient	Age: days	Weight: Kg.	Height: cm.	Surface area: sq. m.	Hematocrit	Blood urea nitrogen: mg. %	Urea clearance: (C _M) cc./min.		Glomerular filtration (G. F.) cc./min.		Slope: natural log	Effective renal plasma flow (P. R.) cc./min.		Filtration fraction (100 X G. F./P. R.) per cent	Tubular excretory capacity (TmPAH) mg./min.		Ratio: P. R./TmPAH	Ratio: G. F./TmPAH	Individual periods per 1.73 sq. m.					Plasma level at mid point mg./100 cc.
							per 1.73 sq. m.	absol.	per 1.73 sq. m.	absol.		per 1.73 sq. m.	absol.		per 1.73 sq. m.	absol.			G. F.	P. R.	TmPAH	Urine flow: cc./min.	C _M	
E. D. ♀	54	3.75		.244	30	12.6	44	6.20	84	11.8	.01065				13.3	1.87	6.32	6.32	81		13	4.12	83	59
																			84		14	3.18	68	57
																			87		13	4.67	55	75
B. K. ♂	55	3.5		.237	33	12.0	36	4.94	65	8.9					51	7.0	1.27	1.27	59		50	2.43	99	54
																			71		48	1.83	72	52
																			97		54	2.57	57	56
P. C. ♂	61	5.3		.312		15.5	26	4.70	63	11.4	.0134	321	58	19.6	93	16.8	.68	.68	54	231		1.68	152	4.28
																			69	342		1.21	120	2.07
																			66	301	94	1.44	97	81
																			26		92	6.04		88
																						10.8		
E. J. ♀	63	4.3		.274		15.5	23	3.64	69	10.9	.0079	263	41.7	26.8	41	6.5	6.4	1.67	62	253		1.56	150	5.10
																			48	243		1.25	118	3.95
																			99	292	44.5	1.65	90	3.63
																						1.61		44
																						1.89		42
																						1.00		43
C. P. ♂	75	5.4		.307	34	17.3	39	6.56	62	11.0	.0111				58	10.3		1.07	59		57	2.53	122	74
																			60		56	3.52	98	84
																			66		60	6.24	81	86
																			62			3.21	65	
																						7.33		
J. S. ♀	81	4.15		.266	37	12.9	33	5.08	73	11.2	.0097	211	32.4	29.8					63	211		.68	86	0.37
																			83			.67	71	
D. K. ♀	101	3.8		.246	39	7.8	47	6.68	71	10.1	.0131	354	50.3	20.0	35	4.98	10.1	2.03	81	411		6.48	104	1.82
																			64	287		3.48	84	1.45
																			70	365	39	1.25	65	1.25
																						9.35		75
																						21.4		72
																						16.05		64
L. W. ♀	108	5.1		.299	35	7.3	41.5	7.17	70	12.1	.00595								59			1.42	85	
																			78			2.48	74	
																			74			3.64	66	
C. M. ♂	118	3.6		.243	36	31.9	27.5	3.9	50	7.0	.00828	204	28.7	24.5					51	201		1.12	120	6.8
																			49	206		1.20	84	4.0
J. M. ♂	137	7.2	66	.388	31				96	21.5		480	108	20.7	57	12.8	8.4	1.68	96	464	71	2.68	102	3.44
																			495		43	2.41		3.67
																					58	4.05		109
																						3.92		108
																						3.42		102

Page 1A—Continued

[illegible]

G.F. in cubic centimeters per minute and in column 10 the figures are corrected for surface area. The individual periods are listed in column 21 to show the variability from period to period. It is apparent from these data that the G.F. is very low in the very young infant, averaging about 50 cc./min. per 1.73 sq.m., and slowly rises during the first year, reaching adult values of about 130 cc./min. some time between the first and second year of life. Under six months of age none of the values were within the adult range when corrected for surface area and only a few in the last six months of the first year. In the second year many were found within the adult range, some still below. After the third year all values were found to be within the adult range.

Table I, column 14, shows the actual values obtained for P.F. in cubic centimeters per minute as measured by the clearance of PAH at low blood levels in the same group of infants and children, and column 13 gives the values per 1.73 sq.m. surface area. Figure 2A graphically presents the rate of effective renal plasma flow corrected to standard surface area as the child matures throughout the first year of life, and in Figure 2B the same

data are given for the older children. As in the case of G.F., the P.F. (corrected for surface area) is also low in the young infant, increasing gradually and reaching the average adult value around the second year.

Table I, column 17, lists the actual values obtained in estimating the maximal tubular excretory capacity in these children, and in column 16 the values are corrected for standard surface area. In Figure 3A the Tm_{PAH} in mg./min. per 1.73 sq.m. is shown for the first year of life, and in Figure 3B the same data are given for the older children. It is apparent that there is much greater scatter of the values for Tm_{PAH} than of those for G.F. or P.F. While the lowest figures obtained, around 3 to 15 mg./min. per 1.73 sq.m. surface area, are seen in the youngest infants (under one month of age), relatively high values are obtained in other infants of the same age. Several already have adult values within the first six months. After 15 months of age the values are comparable to adult values with very few exceptions.

On casual inspection the graphic charts of the data for G.F. and P.F. appear similar, as if

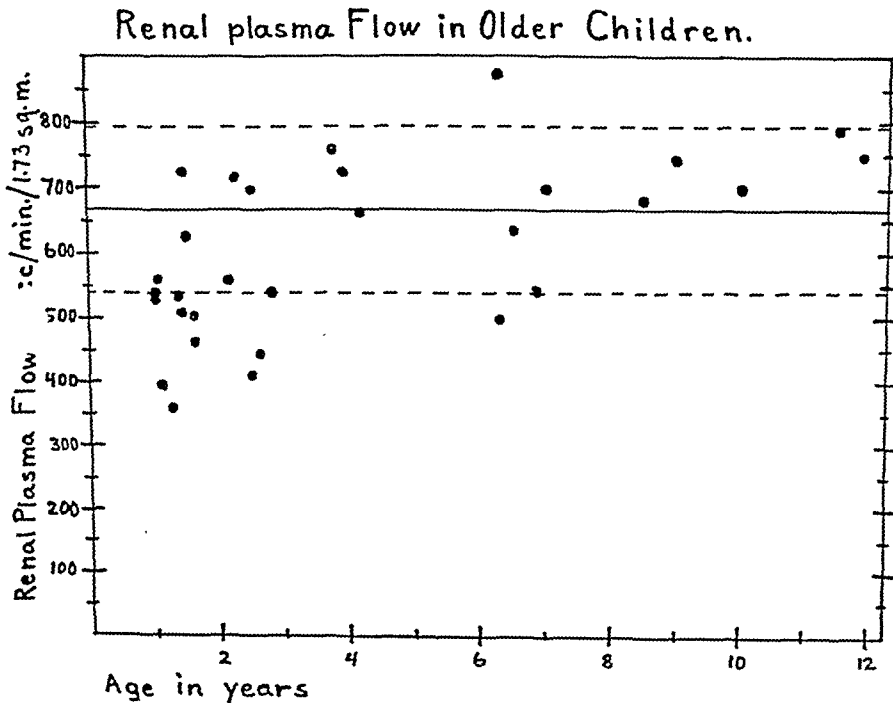


FIG. 2B. EFFECTIVE RENAL PLASMA FLOW IN OLDER CHILDREN
Symbols as in Figure 1A.

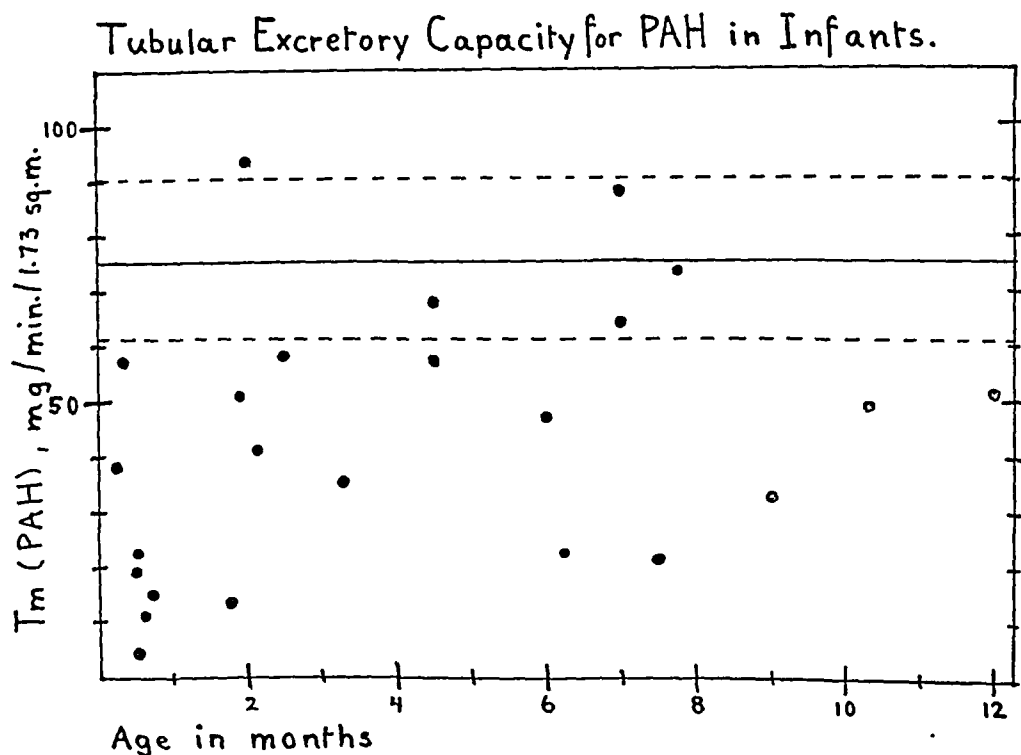


FIG. 3A. MAXIMAL TUBULAR EXCRETORY CAPACITY FOR PAH IN INFANTS
Symbols as in Figure 1A.

the rate of increase of these two physiological factors with aging were identical. Actually, this is not the case as can be seen from the figures on the F.F. ($100 \times G.F./P.F.$) throughout this

age span (Figure 4, upper section). If the rate of increase in these two values were identical, then the F.F. would be a constant value and similar to the average adult value of 19.6%. As can

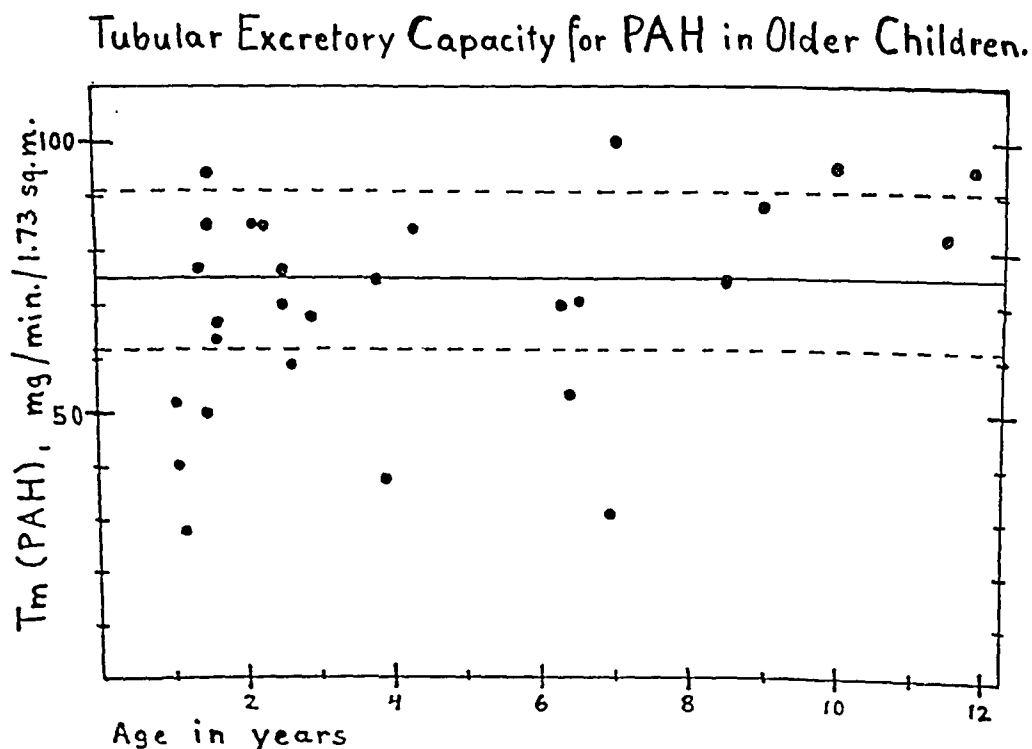


FIG. 3B. MAXIMAL TUBULAR EXCRETORY CAPACITY FOR PAH IN OLDER CHILDREN
Symbols as in Figure 1A.

be seen, this is not the case; for there is a considerable variability of the F.F. throughout the age groups. While several of the values are within the adult range early in life, and occasionally the F.F. is even low in the latter half of the first year, on the average a larger number of higher values are obtained in the first two years. In fact, some high values are found up to the eighth year of life. The average value for the F.F. in children is thus higher than that reported for

adults. It might be stated that the fluctuations rate of P.F. are normally wider than those G.F., being subject to situations which affect general circulation, and might thus account for some of the variations of the G.F./P.F. It must be pointed out that our clearance periods are short and represent "spot" clearances. It remains the possibility of error in estimating P.F. in the very small infants, an error would produce false low values. If the blood

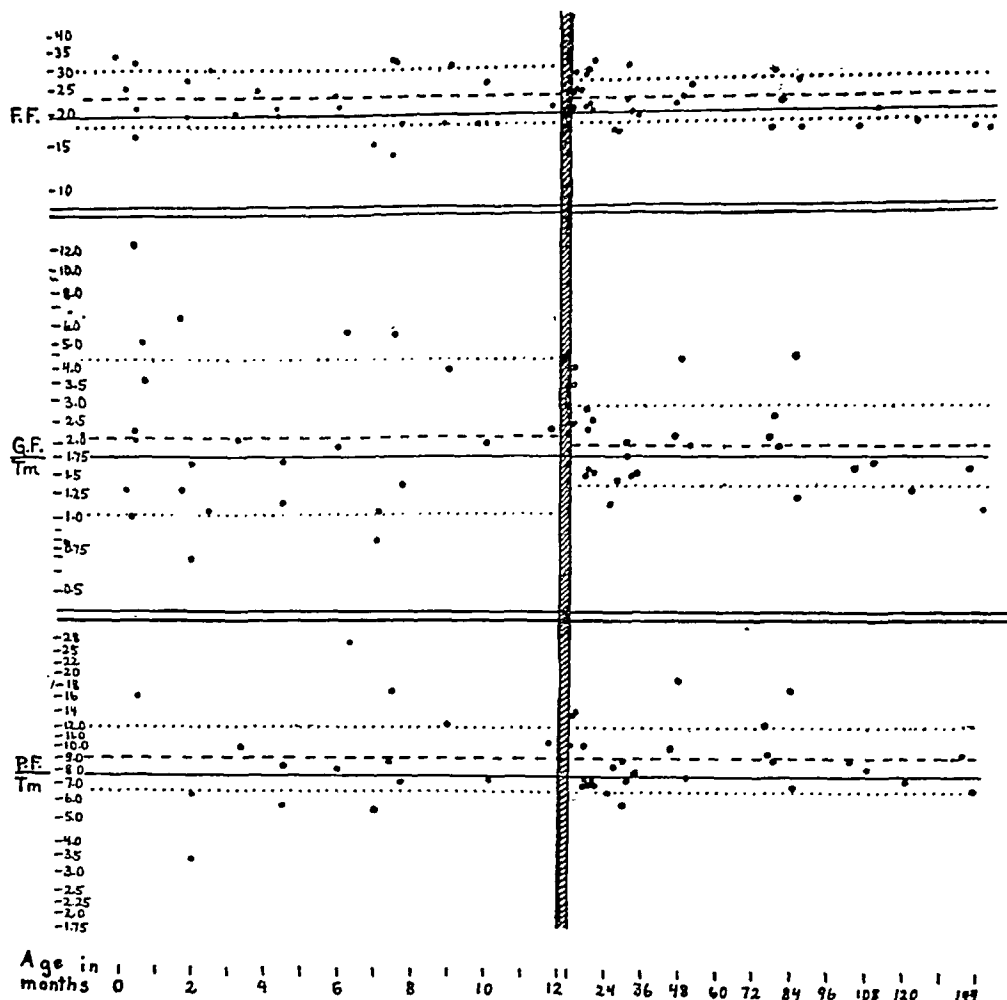


FIG. 4. RATIOS OF RENAL FUNCTIONS TO ONE ANOTHER IN INDIVIDUAL CASES, PLOTTED AGAINST AGE

Upper section: $F.F. (100 \times G.F./P.F.)$.

Middle section: $G.F./T_{mPAH}$.

Lower section: $P.F./T_{mPAH}$.

The ordinate scales are logarithmic, so that equal deviations of the numerator and of the denominator of each ratio from the average will produce equal distances from the line representing the mean. The solid line in each section represents the mean observed in adults (12); the broken line in each section represents the mean observed in the series of children here reported, calculated separately for infants under one year and for children over one year of age. The dotted lines represent one standard deviation from the mean, calculated for the present series.

TABLE IB
Renal functions in children, absolute and corrected for surface area, according to age (months)

Patient	Age: months	Weight: Kg.	Height: cm.	Surface area: sq. m.	Hematocrit	Blood urea nitrogen: mg. %	Urea clearance: (C _u) cc./min.		Glomerular filtration (G. F.) cc./min.		Slope: natural log	Effective renal plasma flow (P. F.) cc./min.		Filtration fraction (100 X G. F./P. F.) per cent	Tubular excretory capacity (TmPAH) mg./min.		Ratio: P. F./TmPAH	Ratio: G. F./TmPAH	Individual periods per 1.73 sq. m.					Plasma level at mid point mg./100 cc.	
							per 1.73 sq. m.	absol.	per 1.73 sq. m.	absol.		per 1.73 sq. m.	absol.		per 1.73 sq. m.	absol.			G. F.	P. F.	TmPAH	Urine flow: cc./min.	Mannitol	PAH	
V. A. ♀	12.2	9.25		.467		10.5	60	16.2	110	29.8	.0133	538	145.3	20.5	51.5				110	535		2.24	124	1.87	
																			110	541		1.24	100	1.63	
M. P. ♂	12.3	11	79	.524		12.0	61	18.5	124	37.6	.0149	529	160	23.5		15.6	10.3	2.4	114	517		2.03	124	2.02	
																			122	467		1.47	94	1.67	
																			138	632	52	1.49	70	1.20	
																					51	2.61		64.5	
																						1.97		48	
E. T. ♂	13	7.5		.405		16.2	64	15.0	136	31.8	.01225	557	130.5	24.0	40	9.37	13.9	3.4	134	546		1.75	67	1.37	
																			67	590		1.77	53.5	1.16	
																			50	434		.97		1.00	
																					37.5	3.02		57	
																					41	2.88		58	
																					42.5	4.17		69	
S. P. ♀	13.7	9.6		.479		11.2	63	17.45	111.5	30.9	.01095	396	110	28.2	28	7.75	14.1	3.99	107	382		2.02	170	2.40	
																			65	390		1.63	146	2.46	
																			111	415	27	1.40	124	1.98	
																			115		29	3.01	75	89	
																					27	2.99		97	
																						4.17		111	
R. M. ♀	15	6.5	76	.387			30	6.72	86	19.2		361	80.7	23.8					89	391		3.08	110	14	
																			29	328		1.57	76	12	
																			26	364		1.20	58	5.5	
T. Z. ♂	17	11.4	76	.516					105	31.4	.0121	510	152	20.6	73	21.8	7.0	1.44	111	506		1.69	155	3.27	
																			105	511		1.72	123	2.40	
																			98	512	70.5	1.31	97	2.09	
																					75.5	2.46		51.5	
																						1.56		35	
J. D. ♂	17	10.5		.508		9.9	65	19.05	139	40.8	.0129	503	148	27.6	50	14.7	10.1	2.73	137	486		1.865	132	2.08	
																			66	533		1.74	112	1.67	
																			141	489		1.545	94	1.52	
																			137		50	3.48	46	79	
																					49	3.26		75	
																						3.25		74	
D. M. ♀	17.5	9.5		.477		9.4	127	35.0	207	57.1	.0164	724	200	28.6	94	25.9	7.7	2.20	208	739		3.81	90	1.14	
																			126	696		2.96	71	1.07	
																			204	737		2.50	56	0.95	
																			209		94.8	5.41		46.5	
																					95.5	5.13		44	
																					92.5	3.92		45.5	

MATURATION OF RENAL FUNCTION IN CHILDHOOD

Continued

MATURATION OF RENAL FUNCTION IN CHILDHOOD	Patient	Age: months	Weight: Kg.	Height: cm.	Surface area: sq. m.	Hematocrit	Blood urea nitrogen: mg. %	Urea clearance: (C _u) cc./min.	Slope: natural log	Effective renal plasma flow (P. F.) cc./min.	Filtration fraction (100 X G. F./P. F.) per cent	Tubular excretory capacity (TmPAH) mg./min.	Ratio: P. F./TmPAH	Ratio: G. F./TmPAH	Individual periods per 1.73 sq. m.	Plasma level at mid point mg./100 cc.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
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J. M. ♂	18	13.6	.607	38	10.6	81	28.4	135	per 1.73 sq. m.	absol.	47.4	135	per 1.73 sq. m.	absol.	219	21.0	66	18.8	7.6	1.50	7.35	1.55	78	121	633	2.19	111	1.44																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												

level of PAH is too high for complete extraction in one circulation through a kidney with a very low Tm_{PAH} , complete extraction of the PAH circulating through the kidney would not occur, thus the PAH clearance in an immature child may be lower than the actual renal plasma flow. It is possible that in some of our small infants, as well as in some of the cases published by others, the PAH load exceeded the tubular excretory capacity, so that falsely low values for P.F. were produced. Data on the PAH extraction ratio in the very small infant are needed to clear this point. The PAH loads were calculated for the 10 youngest infants on whom the plasma flow has been determined in the present study. All the children older than these ten had a Tm_{PAH} well capable of excreting ordinary loads. The infants, E. S. (ten days old), K. C. (19 days old), and A. Y. (19 days old), had loads which would have required a Tm_{PAH} of at least 10 mg./min. per 1.73 sq.m. to assure complete PAH extraction (assuming that complete extraction occurs when $\frac{\text{load (PAH)}}{Tm_{PAH}}$ is less than 0.5); the loads of L. C.

(two days old), R. B. (20 days old), and J. S. (81 days old), were low enough to be excreted with a Tm_{PAH} as low as 2–5 mg./min. per 1.73 sq.m. The remaining four infants in this group had higher loads of PAH, but their Tm_{PAH} was determined and found to be high. Actually, only one infant in the group was observed to have a Tm_{PAH} below 10 mg./min. per 1.73 sq.m.

In order to determine the relative rates of maturation of glomerular and tubular function we have calculated the G.F./ Tm_{PAH} ratio on each child (Table I, column 19 and Figure 4, middle section). The scatter of the data is too wide to draw valid conclusions as to the comparative rates of maturation of these two functions. In general, the G.F./ Tm_{PAH} ratios (normal for adults taken as 1.72) show a wider variation in the infants under two years of age than seen later. Most of our very high values are seen in this younger age group. After two years of age the values are rarely significantly different from adult values.

The amount of renal plasma flow per unit of tubular excretory capacity as measured by the ratio P.F./ Tm_{PAH} is quite variable and evidences

CORRELATION OF UREA CLEARANCE AND FILTRATION RATE

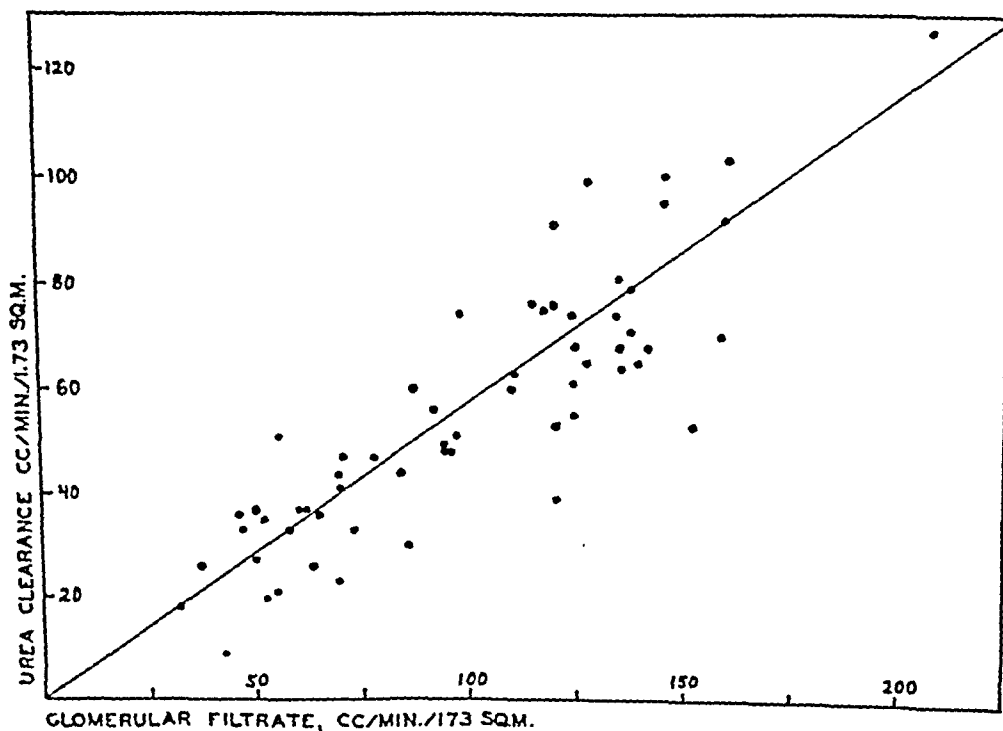


FIG. 5. UREA CLEARANCE, C_m , IN CC/MIN., PLOTTED AGAINST GLOMERULAR FILTRATION RATE

The diagonal line represents a ratio of urea clearance to G.F. of 57.5:100. Each dot represents the average value in an individual child.

the wide variation found in Tm_{PAH} and possibly of the normal fluctuations in P.F. (Table I, column 18 and Figure 4, lower section). In older children the P.F./ Tm_{PAH} ratios are more constantly near the adult value.

The urea clearance values of this same group of children are listed according to age in Table I, columns 8 and 9. The wide variation in urea reabsorption accounts for the variable urea clearance values. Figure 5 shows the relationship between urea clearance and glomerular filtration rate with a correlation coefficient of 0.87. In individual cases the urea clearance varied between 34% and 81% of the mannitol clearance. This great variability has also been observed in adults.

DISCUSSION

It is unfortunate that we could not have collected maturation data in the same individual over the span of childhood,⁵ so that the comparative rates of maturation of the various functions could be determined more precisely. Our data lend themselves only to comparing the average developmental state of one renal function to that of another at a given age.

While, in general, the G.F., P.F. and Tm_{PAH} , when corrected for surface area, reach adult values somewhere around the second year of life, there are exceptions and, as would be expected, these various functions mature at different rates, so that in one child one function has reached maturity while another is still immature and the reverse may occur in a second child. Examination of the simultaneous ratios for these three variables shows certain trends in comparative maturation. It is apparent from the high F.F. (Table I, column 15 and Figure 4, upper section) that G.F. is at a higher level of maturity than P.F. in many of the younger children, though not all. This tendency toward a high G.F./P.F. ratio persists through the second or third year and in some instances later. West and his group (5) have also shown that this ratio is high in most of their infants under two years of

age and Barnett *et al.* (6) in examining premature infants found the F.F. to be even more consistently above that of the normal adult than apparent in the series of full-term and older infants reported here. Elevation of the intraglomerular capillary pressure (as seen in essential hypertension [12]) may exist in infants to account for the high F.F., but this has not been demonstrated. Whether the low serum protein concentration in the small infant is a factor in the increased F.F. can only be conjectured.

The G.F./ Tm_{PAH} ratios (Table I, column 17 and Figure 4, middle section) show a wide scatter. As with F.F., most of the extremely high and low values occurred within the first two years of life. After that time there is less irregularity and only an occasional high value. The few really high values occur in the first eight months of life. However, because of the irregularity of the data one would hesitate to draw a curve to show the trend of relative maturation of G.F. and Tm_{PAH} . The scatter in the ratios is chiefly dependent upon the very variable figures for Tm_{PAH} as the values for G.F. follow a much smoother growth curve. The data obtained by Barnett *et al.* (6) suggest that in premature infants high G.F./ Tm_{PAH} ratio may be a more consistent occurrence. The high figures obtained by West and his co-workers (5) were also observed in the youngest infants. These data suggest that a higher order of maturation of G.F. than of Tm_{PAH} exists in the first months of life but this is by no means constant for in fact, none of our children under six months of age had a G.F. within the adult range, whereas several had a Tm_{PAH} within the adult range at this time.

The irregularity in early life of the other ratios discussed is also apparent in the P.F./ Tm_{PAH} ratio. No definite trend in relative rates of increase in the P.F. and Tm_{PAH} can be noted from the data (Table I, column 18 and Figure 4, lower section). The data of West *et al.* (5) also show greater variability in the younger infants. Whereas, there is a gross correlation between the rate of increase in P.F. and the maturation of Tm_{PAH} , both reaching adult values at about two years of age, the maturation rate of the plasma flow seems steadier and follows a smoother curve. The tubular function of excreting PAH in pre-

⁵ Two infants in the group were studied on repeated occasions: B. K. at 10 and 55 days of age, and E. D. at 22 and at 54 days of age. In both infants the Tm_{PAH} (absolute values) remained stationary over the interim period, while the G.F. increased slightly in B. K. and considerably in E. D.

young infant does not seem to be limited by an inadequate blood supply.

The urea clearances were done simultaneously with mannitol clearances (under the influence of mannitol diuresis); therefore, the urea clearance values are probably higher than would be usual for these children.

It has been suggested (4) that the low G.F. in the young infant is dependent upon the resistance to filtration offered by the visceral layer of Bowman's capsule, which in the small infant is composed of cuboidal cells in contrast to the thin flat cells of the adult membrane (13, 14). The finding of high F.F.'s in many of these infants indicates that, contrary to the above assumption, a higher proportion of the plasma flow is filtered through the infantile glomerulus than through the adult one and, therefore, the thicker cell of the membrane in itself presumably does not reduce the rate of filtration. It is not unreasonable to postulate that the small size and number of capillary loops in the immature glomerulus (13) may account for the decreased blood flow through the glomerulus and thus the decreased G.F. The smaller number and size of capillary loops also offer a smaller filtering surface than the glomerular tufts of the adult, although in relation to the volume of the capillary bed (which influences the blood flow) the filtering surface in these immature glomeruli may be larger than in mature glomeruli. Whether the anatomical difference is the total or even a prime factor responsible for the low G.F. is not known. It is interesting, nevertheless, that both the functional maturation and the anatomic maturation of the kidney glomerulus occur at about the same time, around the second or third year of life (14). Low blood pressure in the small infant could possibly influence the G.F. but G.F. increases over a period of several months when the arterial blood pressure is more or less constant, indicating a lack of correlation between these two. Salmi (15) and Taussig (16) have shown that the arterial blood pressure in infancy changes very little from one week of age until four years of age. The anatomical fact that throughout childhood, and particularly in infancy, the ratio of the renal cortex to the medulla is lower than in the adult (14) might lead us to expect a low G.F./T_{MPAH} ratio. However, our figures do not substantiate such an expectation; an indication

TABLE II

Glomerular filtration rate related to various body measurements in individual patients, arranged according to age

Patient	Age	G. F., cc./min. per				
		Wt.	Ht.	S.A.	Av. kidney wt.	Av. B.M.R.
	Mo.	Kg.	cm.	sq. m.	Gm.	cal./hr.
L. C.	1/15	2.15	0.103	24.8	0.224	1.03
R. S.	1/4	1.87	0.123	27.1	0.262	1.1
E. S.	1/3	2.14	0.126	30.3	0.268	1.1
B. K.	1/3	2.35	0.147	33.5	0.313	1.25
J. K.	1/2	1.97	0.104	26.6	0.213	0.88
B. M.	1/2	1.41	0.103	21.4	0.215	0.9
G. K.	1/2	1.89	0.135	28.9	0.28	1.17
K. C.	2/3	2.58	0.137	35.2	0.28	1.17
A. Y.	2/3	2.31	0.13	32.4	0.27	1.15
R. B.	2/3	1.33	0.08	18.5	0.16	0.65
E. D.	3/4	2.20	0.125	31.8	0.25	0.95
E. D.	1.8	3.14	0.207	48.4	0.39	1.3
B. K.	1.8	2.54	0.156	37.5	0.29	0.99
P. C.	2.0	2.15	0.197	36.6	0.37	1.14
E. J.	2.1	2.54	0.188	39.8	0.35	1.10
C. P.	2.5	2.04	0.187	35.8	0.33	1.0
J. S.	2.6	2.70	0.188	42.1	0.34	1.0
D. K.	3.3	2.66	0.165	41.1	0.28	0.77
L. W.	3.5	2.37	0.195	40.5	0.33	0.90
C. M.	3.9	1.94	0.111	28.8	0.18	0.5
J. M.	4.5	2.98	0.326	55.4	0.55	1.41
E. S.	4.5	2.47	0.275	45.1	0.44	1.15
R. M.	6	3.18	0.24	54.5	0.36	0.91
A. G.	6.2	4.56	0.29	73.9	0.44	1.10
L. T.	7.0	1.59	0.19	30.0	0.27	0.69
B. S.	7.3	2.90	0.33	54.4	0.48	1.22
K. M.	7.3	3.46	0.42	66.2	0.60	1.54
H. B.	7.5	4.15	0.454	77.5	0.66	1.7
K. T.	7.6	2.96	0.34	56.0	0.49	1.25
D. M.	8.8	2.06	0.25	39.9	0.35	0.90
R. L.	9.0	3.68	0.45	71.6	0.61	1.6
J. F.	10.0	2.98	0.305	55.5	0.41	1.1
J. L.	11.7	3.92	0.36	71.6	0.47	1.3
V. A.	12.2	3.22	0.38	63.8	0.48	1.35
M. P.	12.3	3.42	0.475	71.9	0.61	1.7
E. T.	13	4.24	0.40	78.5	0.50	1.4
S. P.	13.7	3.22	0.39	64.5	0.48	1.3
R. M.	15	2.95	0.25	49.6	0.28	0.8
T. Z.	17	2.76	0.413	60.9	0.44	1.2
J. D.	17	3.90	0.49	80.5	0.57	1.6
D. M.	17.5	6.00	0.68	119	0.78	2.2
J. M.	18	3.49	0.57	78.0	0.65	1.8
D. T.	19	2.78	0.37	56.6	0.37	1.0
A. B.	19	3.69	0.58	82.0	0.65	1.8
I. R.	25.7	2.60	0.33	53.0	0.31	0.86
R. S.	27	3.41	0.37	69.5	0.39	1.1
K. E.	30	3.41	0.39	72.0	0.40	1.1
A. S.	30	4.05	0.53	88.0	0.53	1.5
D. G.	31	2.20	0.36	50.3	0.375	1.05
E. M.	34	2.40	0.41	59.0	0.43	1.2
S. C.	45	3.79	0.675	91.5	0.66	1.9
J. D.	47	4.10	0.58	92.0	0.53	1.6
J. C.	50	3.74	0.62	93.0	0.59	1.7
H. M.	75	3.06	0.64	84.3	0.63	2.0
M. W.	76	3.39	0.53	80	0.525	1.6
M. T.	78	2.76	0.47	77.8	0.41	1.3
J. K.	83	3.32	0.55	80	0.53	1.7
C. W.	84	2.76	0.49	69.5	0.475	1.5
A. R.	102	2.43	0.53	68.0	0.49	1.65
C. M.	108	3.26	0.62	84.5	0.55	1.9
B. T.	120	2.75	0.505	69.5	0.43	1.6
D. M.	138	2.95	0.51	74.0	0.32	1.3
M. J.	142	2.42	0.57	69.4	0.46	1.9
Adult		2.0	0.8	76	0.44	2.0

that even the mass of tissue in the early months of life is not closely correlated with function.

Throughout these calculations of renal clearance we have correlated the data collected in the infants and children to adult values using surface area as the basis for comparison, as the principle of correlating renal function to surface area has been widely accepted (5, 17-19). Since this may not be the best point of reference, we have also given the absolute values. In order to determine if surface area is the best basis of comparing the G.F. in the growing child to that of the adult, we have related in Table II the G.F. (in cubic centimeters per minute) at different ages throughout childhood to several different measurements of reference: such as (1) body weight in Kg., (2) body height in cm., (3) body surface area in sq.m., (4) average kidney weight in Gm., and (5) average basal metabolic rate in calories per hour, and have compared these ratios to the adult values. The values for average kidney weight in the different age groups were obtained from the data of Peter (14), and the average values for basal caloric expenditure per hour were obtained from Washburn and Iliff (20). It is apparent that when body weight is used as the measurement of reference, the data are very irregular. In many of the smallest infants the values are above the adult range and after six months of age they are considerably above this level with a gradual decline of the values from a high point during the second and third years of life. When body height is used as reference, there is a gradual increase in the G.F. with growth and the irregularity is less marked than with body weight. However, under these circumstances the values in older children are still much below adult values and this seems unreasonable in the face of other evidence of renal functional maturity in this age group. When surface area is used as standard, occasional adult values are reached around six months of age but the values are not consistently in the adult range until about the second year. Using basal caloric expenditure as reference, there is little change throughout the first six months. After this age the trend is irregularly upward. This increase is slower than the rise seen when surface area correction is used, as might be expected considering the fact that children have a higher basal metabolic rate per sq.m. surface area than adults.

When kidney weight is used as reference, there is a gradual increase of the G.F. per unit of kidney mass, with maturation being reached between the fourth and fifth months of life. After this age and throughout childhood the values more closely parallel the adult values than when any other measurement of reference is used. This is the more remarkable since the values for kidney weight and basal metabolic rate at different age levels represent average values obtained from the literature, whereas, the weight, height, and surface area were measured in the individual patients, who in many instances deviated considerably from average standards.

It has been shown that the mannitol/inulin clearance ratio may be less than one (21). This fact does not invalidate the above data since the same procedure (mannitol clearance) was used in all the age groups and the values were compared to adult values obtained with the same substance (12).

SUMMARY

1. The glomerular filtration rate, effective renal plasma flow, tubular excretory capacity for PAH and urea clearance have been measured in 63 normal infants and children between the ages of two days and 12 years in order to determine the maturation rate of these individual renal functions.

2. In general, when corrected to standard adult surface area, the clearance values were lowest in the smallest infants and gradually rose to reach adult values around the second year of life. Maturation was most rapid in the first six months, then proceeded more slowly. The youngest child in whom all functions were within the adult range was seven months of age. The average child, however, did not show complete maturation of all functions studied before the end of the second year, although individual functions, particularly TM_{PAH} , were often found to be mature at a much younger age.

3. G.F. was found to be closely correlated to adult values after the first few months of life when kidney weight is used as the basis for comparison. When body weight and height are used as the basis for comparison, the data are very variable and show no regular maturational trend. When the surface area is the basis of reference, the maturation rate seems slower than when kidney weight is used. Since in the literature the surface area

is most commonly used as the basis for comparison with adult values, our graphic charts have been constructed using surface area correction.

4. Simultaneous ratios of the various functions were calculated to show relative rates of maturation. In general, it might be said that the ratios show wider variability in the early part of life than later on. The data indicate that these individual functions develop at irregular rates; one function might reach maturity in a given child earlier than another, and in a second child the reverse may occur.

a. There is a tendency toward high filtration fractions (G.F./P.F.) in the early months of life which continues through the second and third years.

b. The rate of Tm_{PAH} maturation is extremely irregular, resulting in great irregularity in the ratios in which Tm_{PAH} is involved.

c. Several of the children under two years of age had a rate of P.F. closer to the adult range than was the Tm_{PAH} (high P.F./ Tm_{PAH} ratio) but this high ratio was also occasionally seen in older children. In most of the determinations even in the very young infants the P.F./ Tm_{PAH} ratio was close to adult values to show roughly parallel rates of maturation.

d. The various ratios estimated would suggest that there is a tendency for G.F. to be more mature in the youngest infants than the other functions measured.

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THE CEREBRAL BLOOD FLOW IN MALE SUBJECTS AS MEASURED BY THE NITROUS OXIDE TECHNIQUE. NORMAL VALUES FOR BLOOD FLOW, OXYGEN UTILIZATION, GLUCOSE UTILIZATION, AND PERIPHERAL RESISTANCE, WITH OBSERVATIONS ON THE EFFECT OF TILTING AND ANXIETY¹

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The nitrous oxide technique for the semiquantitative measurement of cerebral blood flow devised by Kety and Schmidt (1, 2) has made it desirable to restudy the cerebral circulation in man in various physiologic states. By measuring arterio-internal jugular differences of oxygen and glucose, the relation between changes in cerebral blood flow and cerebral metabolism can be observed. The purpose of this report is to record our findings in the use of the nitrous oxide technique in 33 observations on 20 normal young men; in the comparison of certain cerebral metabolic functions on 14 normal young men in the supine and erect postures; and in observations on the effects of apprehension on the cerebral circulation.

METHOD

Seventeen of the 20 subjects were normal, male medical students; the other three (W. Mc., D. M., and R. H. in Table I) were hospital patients who showed no evidence of disease. All the subjects were studied in the fasting state. Following local procainization, a 19 gauge, 6.5 cm. needle was inserted into the right internal jugular bulb in the manner described by Myerson (3), and an arterial needle was inserted in the right femoral artery. Following this the patient was allowed to lie quietly for 40 to 50 minutes before the procedure was continued.

The essential nature of the experiments ascribed in all theoretical details to the nitrous oxide method described by Kety and Schmidt (1, 2), but the mean arterio-venous nitrous oxide difference for the 10 minute period was measured differently. Instead of drawing five separate, simultaneous samples from the artery and vein for the plotting of the nitrous oxide curves, simultaneous continuous samples (called integrated samples) were drawn from the artery and vein into heparinized, oiled syringes

at the rate of 0.25 ml. every 7.5 seconds for the entire 10 minute period of gas inhalation. That this can be done accurately was demonstrated by drawing similar integrated samples simultaneously from the two femoral arteries in four subjects while the subject breathed the nitrous oxide mixture; the mean nitrous oxide content of these samples were identical in each subject. When the collection of the integrated samples is complete, the gas is continued for another 20 seconds while simultaneous samples of 6 ml. each are drawn from the artery and vein. These are used for the determination of the level of the arterial and venous nitrous oxide contents, respectively, at the end of 10 minutes of inhalation. The venous sample represents the cerebral nitrous oxide concentration per gram of brain, since after 10 minutes the interjugular blood and brain nitrous oxide should be in equilibrium and the solubility coefficient for nitrous oxide between blood and brain has been shown to be about 1.0 (4). The arterial sample serves as an internal check on the accuracy of the procedure. It should closely agree with the venous sample at that point, since in normal subjects the arterial curve has almost always flattened out at 10 minutes, and equilibrium between arterial and cerebral venous blood is approaching; lack of agreement indicates technical error, poorly-placed venous needle, unsteady gas administration, any of which would invalidate the observation. In our experience, the agreement in each instance has been close, the greatest variation being 0.44 volumes % nitrous oxide in a subject with relatively slow flow, and the average variation in 49 terminations being 0.24 volumes %. The nitrous oxide mixture is always given in such excess that only an extremely gross leak could alter the determination. It is felt that this is probably of small importance actually, for our figures have not varied in subsequent patients, in whom a self-retaining mask was used. The potential advantages of the integrated sample technique will be dealt with at the end of this section.

The blood samples were placed in ice immediately after being collected, the usual precautions having been taken to assure the absence of air bubbles. The pH of the integrated arterial sample was measured with a glass electrode by means of a Beckman pH meter immediately after each blood flow.

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The subject was allowed to remain supine for 20 to 25 minutes following the first blood flow; then the table was tilted so that the subject stood erect at an angle of 65° to the horizontal. In this position the weight was supported partially by the legs and partially by leaning against the table. When the subject's blood pressure and pulse stabilized, and he showed no signs of ensuing syncope, the same procedure of gas inhalation and blood withdrawal was performed. The patient was then put supine, and if he was not uncomfortable or tired, a third flow was done after a wait of 25 minutes.

The arterial pressure was measured by auscultation every two minutes during the supine flows and every minute during the erect. In both positions measurement was always done with the subject's arm held at approximate heart level. From these readings average mean pressures were computed by adding one-third of the pulse pressure to the diastolic pressure. This method is in use in other laboratories, and observations here have shown that the mean pressure calculated in this way is about the same as that measured by the Hamilton manometer. In 91 observations in this laboratory the mean pressure calculated by adding one-third of the pulse pressure to the diastolic pressure on the manometer tracings varied on the average of 3 mm. Hg from the mean pressure calculated by the planimeter method. In order to have altered the accuracy of our comparisons of cerebral vascular resistance in the supine and erect posture, the error in mean arterial pressure would have had to be in the neighborhood of 25 mm. Hg, and consistently so. The pulse rate was measured every two to three minutes. Internal jugular venous pressures were measured by means of a spinal fluid manometer; the zero point was considered to be the needle level.

Arterial and venous samples were drawn simultaneously before and after each flow for glucose determinations, which were done by Nelson's photometric adaptation of the Somogyi method (5). The average of the two A-V glucose differences for each flow is used as the result.

In seven instances, single samples, drawn before and after the flows, were analyzed for oxygen by the Van Slyke manometric method (6) and averaged. In all other instances, oxygen determinations were made on the integrated blood samples by means of the Beckman spectrophotometer, using the method described by Hickam and Frayser (7).

The blood samples were analyzed for nitrous oxide by Kety's modification (2) of the original method of Orcutt and Waters (8). Here again certain modifications were made to suit our needs. The determinations were done on two different machines by two technicians, serving as checks on each other. The results were discarded if the calculated cerebral blood flows from the two machines did not agree within 10%. In all but three instances the flows agreed within 7%. Five ml. blood samples were used in the analyses on the integrated arterial and venous samples. This was done in order to give a larger volume of gas and thus render the difference of the manometer readings between the arterial

and venous samples 2.5 times as great as with 2 ml. samples, and accordingly reduce the percentage error of the individual determinations. The mean arterio-venous nitrous oxide difference must be determined carefully, for a variation in this difference of 0.01 volumes % is equivalent to a variation of 0.09 volumes % in the final venous nitrous oxide sample. Samples of internal jugular blood, drawn immediately before each flow, were used for the blank determination. The difference between the blank samples on the same individual were negligible; it never varied more than 0.25 volumes %. A sample determination is as follows:

Blank = 1.23 volumes %,

Integrated arterial sample = 5.34 volumes %,

Integrated venous sample = 4.53 volumes %,

Mean arterio-venous nitrous oxide difference for 10 min. = 0.81 volumes %,

Single venous nitrous oxide sample at 10 min. (V_t) = 5.05 volumes %,

Single arterial nitrous oxide sample at 10 min. (A_t) = 5.19 volumes %.

The cerebral blood flow can be calculated simply from these data:

$$CBF = \frac{5.05}{0.81 \times 10} \times 100 = 62 \text{ ml./min./100 gm. brain.}$$

The factor 100 is to convert the answer to units of per 100 gm. of brain, to be consistent with other data in the literature. The factor 10 converts the mean arterio-venous nitrous oxide difference per unit time (one minute) into the total mean arterio-venous nitrous oxide difference for the entire 10 minute period. The denominator is thus equivalent to Kety's expression $\int_0^{10} (A-V) dt$. In 49 complete cerebral blood flow analyses done by two technicians on different Van Slyke machines, on normal supine subjects, the following mean figures were obtained for the integral parts of the formula: $\int_0^{10} (A-V) dt = 7.11$ volumes per cent; $V_t = 4.68$; $A_t = 4.92$ with standard errors of 0.30, 0.05, and 0.09 respectively.

In this study the direct measurement of the mean arterio-venous nitrous oxide difference has the following advantages over the calculations of the mean difference from five separate samples: (1) Since only five Van Slyke analyses (and checks) are required for each blood flow (11 analyses [and checks] are required for the original method), several blood flows can be done the same day without straining the laboratory facilities. (2) It seemed more accurate to allow the body to integrate the samples than to draw curves through individual points. (3) Smaller quantities of blood can be used if necessary.

It is important to keep in mind that the nitrous oxide method measures only the mean cerebral blood flow over the 10 minute period of gas inhalation. Results obtained when the flow might be changing must be interpreted in that light; otherwise erroneous conclusions may be made. For example, if a subject had an abnormally rapid blood flow during the first five minutes of the observation, the nitrous oxide content of the cerebral

venous blood may have reached or approached equilibrium with the nitrous oxide concentration of the brain at five minutes rather than 10 minutes, so that even if the blood flow fell to a very low level during the second five minutes, the analyses of the bloods would show a small mean arterio-venous nitrous oxide difference, and the flow would be calculated as being rapid. In studies on erect subjects, it is possible that the cerebral blood flow was falling progressively during the entire time, in which case an accurate mean flow for that period could be calculated. If, however, the flow was stable for the first six to eight minutes, and then fell rapidly during the last two to four minutes, the recorded blood flow would be calculated falsely high. It would seem, therefore, that if there is a mistake in our comparison of the supine and erect flows, it is that the average flow in the erect posture was actually lower than we have recorded it, and that the difference between supine and erect flows is even greater than our figures indicate. This can be checked by drawing cerebral arterio-venous oxygen differences before and after the flow, which we did in five instances, as mentioned in the results. If the flow falls progressively the integrated arterio-venous oxygen difference will be equal to the average of those drawn before and after. In our five subjects this was true. The formulae for the calculation of various metabolic functions discussed in this paper are given below:

$$\text{Cerebral O}_2 \text{ consumption (CMR O}_2\text{)} = \text{CBF} \times \frac{A - \text{VO}_2}{100}$$

in ml. O₂/min./100 gm. brain.

$$\text{Cerebral glucose consumption (CMR gl.)} = \text{C} \times \frac{A - \text{V glucose}}{100} \text{ in mgm. glucose/min./100 g brain.}$$

Cerebral vascular resistance

$$(\text{CVR}) = \frac{\text{mean arterial pressure}}{\text{CBF}}$$

in mm. Hg/ml. blood/100 gm. brain/min.

RESULTS

Normal Values. The essential data are given in detail in Tables I and II. The cerebral blood flow supine varied from 50 to 103 ml./min./100 g. brain. Our mean figure for cerebral blood flow is 65 ml./min./100 gm. brain (standard error 2.1) this is a significant difference statistically from the mean of 54 (standard error 2.1) reported by Kety (2), as indicated by the small standard errors of the two groups. The reasons for this difference are not clear: The average age of the subjects or differences in the techniques in the two laboratories may be responsible. The cerebral arterio-venous oxygen differences supine varied from 4.4 to 7.6 volumes % with a mean of 6.0, the mean reported by Kety is 6.3. The cerebral oxygen utilization ranged from 3.1 to 5.2 . .

TABLE I
Cerebral metabolic functions in normal individuals in the supine and erect postures

Subject	Age	Surface area	Average pulse rate	Angle tilted	Apprehension	Cerebral blood flow ml./min./100 gm. brain			A-V O ₂ diff. vol. %		
						Sup.	Er.	Sup.	Sup.	Er.	Sup.
J. G.	27	sq. M.	90		0-4+	103		93	4.37*		4.63*
R. K.	24	1.92	70		2+	50			7.23*		
J. C.	24	2.01	60	65°	0	64		65	7.00*	6.33*	6.34*
L. S.	24	1.80	75	65°	0	78	51	56	5.53	7.01	5.42
R. M.	29	1.81	68	65°	0	62	53	59		8.83*	8.84*
H. D.	27	1.82	76	65°	2+	61	48		5.85	7.83	7.19*
D. M.	31	1.98	74	65°	2+	52	40		5.91	9.78	
G. C.	23	1.86	64	65°	0	65		71	6.05	6.66	6.18
J. C.	25	2.00	76		1+	66			5.08	6.41*	
D. D.	22	2.24	60	45°	0	63	56	58	5.13	6.13	5.10
P. S.	22	1.73	86	50°	2+	62	53	59	5.99	7.20	5.68
W. B.	27	2.14	72	40°	1+	68	42	54	6.07	9.81	5.95
J. T.	26	2.03	80	45°	0	61	45	56	6.33	7.43	6.25
J. R.	23	1.93	60	65°	0	97	81	78	5.09	5.35	4.80
J. S.	26	1.72	72	65°	0	54	42	63	5.75	6.65	4.95
J. H.	28	1.89	64	65°	0	65	57	61	5.43	6.12	5.67
T. C.	21	2.04	73	50°	2+	58	51		6.74	9.37	7.64
W. Mc.	19	1.69	100		2+	55			7.60*		
D. M.	18	2.19	60		0	62			6.30*		
R. H.	36	1.86	70		0	56			6.70*		
Mean						64.9	51.6	64.4	6.01	7.39	5.98

Sup. = Supine.

Er. = Erect.

* These figures are the averages of O₂ determinations on two individual samples, one drawn before the blood flow and the other at the end of the flow. All others are determinations on integrated samples, collected throughout the procedure, as described in the text.

TABLE I—Continued

Subject	A-V glucose diff. mgm. %			Cerebral O ₂ utilization ml. O ₂ /min./ 100 gm. brain			Cerebral glucose utilization mgm. glucose/min./ 100 gm. brain			Effective mean cerebral arterial pressure mm. Hg			Cerebral vascular resistance mm. Hg/ml. blood/ 100 gm. brain/min.		
	Sup.	Er.	Sup.	Sup.	Er.	Sup.	Sup.	Er.	Sup.	Sup.	Er.	Sup.	Sup.	Er.	Sup.
J. G.	6		8	4.50		4.31	6.18		7.43	94		80	0.91		0.86
R. K.	10			3.62			5.00			86			1.70		
J. C.	9	11	9	4.48		4.05	5.75		5.84	86	56	90	1.34		1.35
L. S.	9	12	7	4.31	3.58	3.03	7.00	6.13	3.94	87	46	78	1.11	0.90	1.39
R. M.					4.68	5.18				70	49	71	1.13	0.93	1.20
H. D.	14	8	13	3.57	3.77		8.54	3.84		82	52		1.34		1.08
D. M.	9	7	11	3.08	3.90		4.68	2.80		100	50		1.93	1.25	
G. C.	11	11	11	3.93		4.32	7.15		7.58	73	46	74	1.12		1.04
J. C.	12	7		3.35			7.92			81			1.16		
D. D.	8	13	8	3.33	3.43	3.06	5.20	6.72	4.62	91	61	89	1.40	1.09	1.48
P. S.	13	13	16	3.71	3.82	3.35	8.06	6.90	9.46	72	51	75	1.16	0.96	1.27
W. B.	12	14	10	4.12	4.12	3.21	8.16	5.90	5.92	90	60	79	1.32	1.43	1.46
J. T.	9	8	10	3.86	3.35	3.50	4.27	3.60	5.60	92	73	92	1.51	1.62	1.64
J. R.	7	11	7	4.93	4.33	3.75	6.30	8.50	5.08	85	62	84	0.88	0.77	1.07
J. S.	12	9	8	3.11	2.80	3.12	6.75	4.00	4.72	78	57	77	1.44	1.35	1.22
J. H.	9	11	11	3.53	3.49	3.46	5.85	6.27	6.71	80	59	79	1.23	1.03	1.29
T. C.	11	14	12	3.91	4.72		6.10	7.15		84	50	87	1.45	0.98	
W. Mc.				4.18						92			1.67		
D. M.	7			3.78			4.34			90			1.45		
R. H.	9			3.74			5.60			70			1.25		
Mean	9.6	10.6	10.0	3.84	3.83	3.69	6.27	5.61	6.08	84.5	55.1	81.2	1.33	1.12	1.27

oxygen/min./100 gm. brain. The mean figure for cerebral oxygen utilization obtained here was 3.8 ml. oxygen/min./100 gm. brain, while that obtained in Kety's lab was 3.3. The difference is due to the difference in the figures for cerebral blood flow. The other values are quite similar to those reported from other laboratories (9-12).

The cerebral vascular resistance in the supine position ranged from 0.86 to 1.93, with a mean of 1.31. The cerebral vascular resistance has been given as the quotient of the $\frac{\text{mean arterial pressure}}{\text{cerebral blood flow}}$,

disregarding the internal jugular pressure. Only preliminary observations on internal jugular pressure were made; further studies are now in progress and will be reported at a later date. The available measurements indicate that the average fall in internal jugular pressure on assuming the erect posture is 60 mm. water.

The cerebral arterio-venous glucose differences supine ranged from 6 to 16 mgm. %, and the cerebral glucose utilizations varied from 3.9 to 9.5 mgm. glucose/100 gm. brain/min. The ratios of cerebral arterio-venous glucose difference to ar-

TABLE II
Cerebral metabolic functions in normal persons in the supine posture

	No. of determinations	Mean	Std. dev.*	Std. error†	Coeff. of variation
CBF (ml./min./100 gm. brain)	32	64.7	12.1	2.14	19%
A-V O ₂ diff. (vol. %)	33	6.02	0.81	0.14	13%
A-V glucose diff. (mgm. %)	32	9.94	2.39	0.42	24%
CMR O ₂ (ml. O ₂ /min./100 gm. brain)	31	3.79	0.55	0.09	13%
CMR glucose (mgm. glucose/min./100 gm. brain)	29	6.20	1.41	0.26	22%
Mean arterial pressure (mm. Hg)	33	83	8.32	1.45	10%
Cerebral vascular resistance (mm. Hg/ml. blood/ 100 gm. brain/min.)	32	1.31	0.24	0.04	18%
A-V glucose/A-V O ₂ ratio	31	1.67	0.353	0.06	21%

$$* \text{ Standard deviation } = s = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

$$† \text{ Standard error } = \frac{s}{\sqrt{n}}$$

TABLE III

Relationship of cerebral blood flow to apprehension

Subject	Apprehensive Cerebral blood flow	Subject	Not apprehensive Cerebral blood flow
	ml./min./sq. M. body surface		ml./min./sq. M. body surface
J. G.	700	J. C.	453
R. K.	370	L. S.	521
H. D.	470	R. J.	472
D. M.	368	G. C.	512
P. A.	494	D. D.	382
W. B.	399	J. T.	407
W. McL.	452	J. R.	638
J. C.	462	J. S.	480
T. C.	398	J. H.	467
		D. M.	397
		R. H.	422
Mean	457	Mean	468

terio-venous oxygen difference varied from 1.10 to 2.46, a mean of 1.67. The variations are probably related primarily to the method of glucose determination. It is interesting that, although the expected ratio is 1.34, the figure obtained in this study corresponds closely to that of 1.62 obtained by Gibbs *et al.* (12) in 50 determinations on 25 schizophrenic and epileptic patients. There is no evidence for altered cerebral metabolism in such subjects (13). There is no indication that inhalation of 15 per cent nitrous oxide alters this figure. Since our present knowledge of normal brain metabolism is incomplete, as recent studies (14) have demonstrated, it may well be that the ratio of 1.34, anticipated from the chemical equation of the complete breakdown of glucose to carbon dioxide and water is inaccurate. Though oxygen: carbon dioxide ratios have indicated a cerebral R.Q. of about 1.00, it is entirely possible that other mechanisms, such as the partial oxidation of glucose to pyruvic and lactic acids, and the conversion of

carbohydrate to fat, may fortuitously place the figure at 1.00.

Apprehension. The effect of apprehension on cerebral blood flow is summarized in Table II. The criteria used to determine apprehension were (1) clinical observations, (2) rapid pulse, and (3) elevated systolic pressure. Those subjects who were considered to be apprehensive had a range of cerebral blood flow from 370 to 700 ml./min. sq. M. body surface; those who were not considered to be apprehensive had a range of 382 to 638. There is no significant difference between the means of these two groups.

Posture. The effects of changing the subject from the supine to the erect position are summarized in Table IV. The mean cerebral arterial pressure in the erect position was calculated by subtracting from the mean erect brachial arterial blood pressure the vertical distance in mm. Hg from the second rib to the mid-cerebrum. This can easily be calculated since the patient's angle to the horizontal plane is known. Although we did not make direct measurements of the carotid pressure in the erect position, it is felt that the application of hydrostatic laws to the circulation justifies this assumption. Measurements of common carotid arterial pressure with the subjects in a vertical head-up position by Loman (15) showed a mean fall of 20 mm. Hg in 23 observations. This figure closely corresponds with our calculated one of 22 mm. Hg fall, if it is considered that our subjects were only tilted to 65° and that the mid-cerebrum is about 15 cm. cephalad to the point at which Loman and his group were measuring.

The cerebral blood flow in the erect position varied from 40 to 81 ml./min./100 gm. brain, with

TABLE IV

Alterations of cerebral metabolic functions on changing from the supine to the erect posture

	No. of Comparisons	Mean		P value*	Change %
		Supine	Erect		
CBF (ml./min./100 gm. brain)	13	65.1	51.6	<0.01	-21
A-V O ₂ diff. (vol. %)	13	6.00	7.39	<0.01	+23
A-V glucose diff. (mgm. %)	14	10.10	10.60	>0.5	
CMR O ₂ (ml. O ₂ /min./100 gm. brain)	13	3.84	3.83		
CMR glucose (mgm. glucose/min./100 gm. brain)	11	6.40	5.61	0.2	
Mean Arterial pressure (mm. Hg)	14	84.0	55.0		-34
CVR (mm. Hg/ml. blood/100 gm. brain/min.)	12	1.33	1.12	<0.01	-16
A-V glucose/A-V O ₂ ratio	14	1.76	1.54	0.17	

* P value of 0.02 or less is considered significant.

a mean of 52. This represented a 21% fall in cerebral blood flow over the supine position. This is a highly significant change statistically. Observations reported by Shenkin *et al.* (16) using the nitrous oxide technique showed no fall in cerebral blood flow in five normal persons tilted head-up 20° from the horizontal.

The cerebral arterio-venous oxygen difference in the erect position ranged from 5.4 to 9.8 volumes %, with a mean of 7.4 volumes %. This represents an increase of 23% over the supine position, a highly significant change statistically.

The cerebral oxygen utilization, cerebral arterio-venous glucose difference, cerebral glucose utilization, and $\frac{A-V \text{ glucose}}{A-V \text{ oxygen}}$ ratio did not change significantly.

The effective mean cerebral arterial pressure fell an average of 34% from the supine to the erect position. The vertical distance from the second rib to the mid-cerebrum in our subjects averaged 22 mm. Hg; the average fall in mean auscultatory pressure on standing erect was 7 mm. Hg. The cerebral vascular resistance in the erect position ranged from 0.90 to 1.62, with a mean of 1.12. This represented an average fall of 16% from the supine position, a highly significant change statistically.

Because other studies have pointed out the fall in cerebral blood flow associated with over-ventilation (17) the arterial blood pH was measured after each blood flow. There was no difference between the pH in the supine and that in the erect posture, indicating that the observed alterations in cerebral metabolism during posture change were not related to changes in respiration, insofar as these changes could be measured by the blood pH.

In five instances, cerebral arterio-venous oxygen differences were measured when the subject was first tilted erect and again just before he was put supine, as well as from the integrated samples. In every instance the arterio-venous oxygen difference increased during the period of the flow, indicating the value of the integrated samples for accurate calculation of mean cerebral oxygen utilization.

COMMENT

The values for the various normal metabolic functions of the brain vary over a wide range

from person to person, and these variations are not altered significantly by relating the values to the surface area of the patient. Statistical analysis of available anatomical data of brain weight and surface area (18) reveals that the correlation coefficient of these figures is between 0.45 and 0.50, roughly in the range of correlation of other biological variables. The variations from individual to individual of the values for cerebral metabolic functions seem to be due to two causes: (a) much of the variation is probably real, just as there is known to be individual variations in other physiologic functions, and (b) technical errors are probably partially responsible, as are minor anomalies in the anatomy of the internal jugular circulation. There seems to be no way of predicting at present whether contamination of the jugular blood by a vein draining the subcutaneous and deep tissues of the face would erroneously raise or lower the calculated cerebral blood flow. If arterio-venous shunts in the skin were open and contributed to the contamination, the figure would be falsely high; if the contaminating vein drained only areas of fat, the figure might be falsely low. Studies to clarify this problem are in progress.

The accumulated data on normal individuals seem to indicate that the rate of cerebral oxygen utilization during the time of the experiment is a fairly constant figure in any given person, and that, lacking clinical signs of cerebral dysfunction, the cerebral arterio-venous oxygen difference increases as the rate of blood flow falls. The alterations in cerebral blood flow and cerebral arterio-venous oxygen difference on assuming the erect posture are of similar magnitude (−21% and +23% respectively) but in opposite directions, so that the rate of cerebral oxygen utilization remains the same. The same relationship would be expected to hold for cerebral blood flow and arterio-venous glucose difference, but technical inaccuracies involved in performing the analyses for blood glucose obviate any definite conclusions on this score at present.

The ability to maintain a stable rate of oxygen utilization under most conditions exists in the liver (19) as well as the brain. The kidney, on the other hand, does not behave similarly, but maintains a fairly constant arterio-venous oxygen difference in the face of a considerable fall in blood flow (20). Under certain conditions of duress, the

brain also behaves differently; Kety and co-workers have shown that in insulin hypoglycemia the cerebral blood flow remains stable, while the cerebral arterio-venous oxygen and arterio-venous glucose differences fall strikingly, giving lowered rates of cerebral oxygen and glucose consumption (21).

We have compared this group of normals with a group of similar individuals studied by Stead *et al.* (22), on whom the cardiac index in the resting state was determined. It seemed fair to make this comparison because of these reasons: (a) The subjects were, in both instances, mainly normal medical students and were studied under similar conditions; (b) The average surface area in the two groups was almost identical (1.87 sq.M. in Stead's study and 1.92 sq.M. in this study); the size of the groups was also the same; and (c) the addition of another procedure, such as the measurement of the cardiac output, to that of measuring the cerebral metabolism, would almost necessarily obviate the existence of a basal state in the subjects. Table V summarizes these com-

TABLE V

Relationship of cerebral blood flow and cerebral O₂ consumption to cardiac output and total O₂ consumption

Cardiac index (l./min./sq. M.)*	3.3
Cerebral blood flow (l./min./sq. M.)	0.463
% Cardiac output to cerebral circulation	14%
Total O ₂ consumption (ml./min./sq. M.)*	128
Cerebral O ₂ utilization (ml./min./sq. M.)	27.8
Cerebral O ₂ consumption as percentage of total O ₂ consumption	22%

* Stead *et al.* (22); see text for explanation.

parisons. Our calculations were made on the basis of an average brain weight of 1400 gm. Since we were comparing two different groups, only comparisons of means could be made. The data indicate that approximately 14% of the cardiac output in the resting state is devoted to the cerebral circulation, whereas the cerebral oxygen consumption is about 22% of the total oxygen consumption of the body. If the figures given by Kety and Schmidt (17) for percentage of cardiac output devoted to cerebral circulation and cerebral oxygen consumption as percentage of total oxygen consumption are recalculated using 1.0 as the solubility coefficient of nitrous oxide between brain and blood, the resultant averages of 15% and 19% closely approximate our results. It should be

pointed out that this agreement appears to be fortuitous, for their figures for cardiac output, measured by the ballistocardiogram, are moderately lower than those usually given in measurements done by the direct Fick principle.

The finding that the cerebral blood flow is not altered in apprehension is of considerable physiological interest. Although Table III includes only cerebral blood flow, no correlation was found between apprehension and any aspect of cerebral metabolic function measured in this study. The cardiac output increases about 40% during apprehension (22, 23); it would be interesting to know where the blood goes in this condition. Apparently it does not go to the brain, kidney, or skin.

Although our studies have not revealed a quantitative relationship between alterations in the arterial pressure and cerebral blood flow in the same person, there was a definite fall in cerebral blood flow with the fall in effective cerebral arterial pressure on standing. The significance of the fall in cerebral vascular resistance in the erect posture cannot be interpreted with available data. There are at least two possibilities: (a) The cerebral vessels may actually dilate in response to a fall in arterial pressure or in response to whatever stimuli result from standing erect. Direct observations of the pial vessels of the cat through a Forbes window have shown that a fall in arterial pressure is a strong stimulus for the production of pial vasodilatation (24). (b) The fall in cerebral vascular resistance may be the result of a decrease in pressure on the venous side of the capillaries, since blood in the internal jugular system must certainly run down-hill when the subject is erect.

SUMMARY AND CONCLUSIONS

1. The nitrous oxide method for measurement of cerebral blood flow was used in 33 observations on 20 normal young men in the supine posture. Modifications in the original method are described.

2. Normal figures are given for cerebral blood flow, cerebral arterio-venous oxygen difference, cerebral oxygen utilization, cerebral arterio-venous glucose difference, cerebral glucose utilization, and cerebral vascular resistance.

3. In normal, resting subjects 14% of the cardiac output is devoted to the cerebral circula-

tion; 22% of the total oxygen consumption of the body is cerebral oxygen consumption. The significance of this is discussed.

4. Observations on apprehensive subjects revealed no difference in cerebral metabolic function from those who were not anxious.

5. Tilting normal subjects to the erect posture resulted in a 21% fall in cerebral blood flow, a 23% increase in cerebral arterio-venous oxygen difference, a 34% fall in effective cerebral arterial pressure, and a 16% fall in cerebral vascular resistance.

6. Cerebral oxygen utilization is a relatively constant figure in the normal person under ordinary stresses. Its buffer system is flexible, allowing the cerebral arterio-venous oxygen difference to compensate for relatively large changes in cerebral blood flow.

7. The fall in cerebral vascular resistance in the erect position may be the result of cerebral vascular dilatation or a decrease in pressure on the venous side of the cerebral capillaries.

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SEROLOGICAL STUDIES IN RHEUMATIC FEVER. II. SERUM COMPLEMENT IN THE RHEUMATIC STATE¹

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In the study of rheumatic fever there has been disagreement and confusion as to the level of serum complement (C'). Veil and Bucholz (1), Coburn (2), Rachmilewitz and Silberstein (3), and others have reported a low complement content of the serum, which rises to normal with the subsidence of activity. This has been cited as possible evidence of the occurrence of an antigen-antibody reaction in rheumatic fever, perhaps related to a preceding hemolytic streptococcus infection. Predominantly normal or slightly low levels of C' in rheumatic fever were reported by Kellett and Thomson (4), and more recently by deGara and Goldberg (5). On the other hand, high values during the early period of rheumatic activity and low values later were noted by Hadjopoulos and Burbank (6). In this respect, rheumatic fever did not differ from several other febrile illnesses which they studied. The variety of results obtained by different investigators may be attributed in part to the variety of technics employed, and the lack of a reproducible quantitative method. The purpose of the present study was to reevaluate complement levels in rheumatic fever with the accurate quantitative method developed by Mayer, Osler, Bier and Heidelberger (7, 8), in which the 50 per cent hemolytic unit is determined spectrophotometrically in the presence of adequate concentrations of Mg⁺⁺ and Ca⁺⁺.

METHOD

Details of the analytical procedure as outlined by Mayer *et al.* (8, 9) were carefully observed since this is essential to obtain accurate and consistent results.

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The diluent for all reagents was a barbiturate buffered sodium chloride solution (8) with the addition of 1.0 gm. magnesium chloride and 0.2 gm. of calcium chloride to the 2 liter stock solution. The stock solution was diluted 1 part up to 5 with distilled water to make an isotonic solution of pH 7.3-7.4. Sheep cells were obtained by sterile technic from the external jugular vein of sheep and preserved in sterile Alsever's solution (8). The suspension was kept at +2 to +5° C. and although some hemolysis occurred after three or four weeks, cells preserved in Alsever's solution and washed five times with buffered saline were no more fragile or susceptible to lysis than fresh cells, and yielded the same results as did washed fresh cells. After washing five times, buffered saline was added to the cells until a standardized suspension was obtained. Standardization was performed by lysing exactly 1.5 ml. of the cell suspension with 13.5 ml. of distilled water in a 15 ml. volumetric flask. The resulting solution was read in the Coleman Universal Spectrophotometer at 5500 Å and, when standardized in a 13 mm. square cuvette, gave a value of $D_{5500} = .560$, corresponding to an original cell concentration of about 660,000 cells per cu.mm. or approximately a 2 per cent suspension. The amboceptor was rabbit serum containing antibodies to sheep red cells (Lederle) titrated for minimal hemolytic potency in the presence of an excess of C'. Four minimal hemolytic units of amboceptor, an excess, were employed in each test. The amboceptor was appropriately diluted with buffered saline so that it could be mixed in exactly equal volumes with the standardized sheep cell suspension. The resulting mixture was incubated at 37° C. for 15 minutes. On lysis of 1.5 ml. of the sensitized and standardized sheep cell suspension with 6 ml. of distilled water, a value approximating $D_{5500} = .560$ was obtained. This value, obtained in duplicate, served as the standard for the point of 100 per cent lysis and as a reference for the degree of hemolysis in the experimental tubes.

Blood was obtained from rheumatic and other individuals at the Presbyterian Hospital, Babies Hospital, and the Pelham Home for Children. After clotting at room temperature for about two hours, the blood was centrifuged for 10-15 minutes at 2,000 RPM and the supernatant serum poured off. The serum was frozen immediately and kept in the CO₂ icebox until used. By storing sera in this way, repeated determinations were possible at different times and reproducible results were obtained after storage for seven months. Storage of serum in a mechanical deep freeze unit at -17° C. did

not preserve the C' activity. A loss of 25 to 33 per cent of the original C' activity of the sera occurred in one week at this temperature.

Tests were carried out in centrifuge tubes of 40 ml. capacity to allow thorough mixing. The addition of reagents to each tube was made with analytical precision in the following order: Exactly 1.5 ml. of the standardized sensitized sheep cell suspension, buffered saline to make a final volume of 7.5 ml. after all reagents are in the tube, and serum, diluted 1 to 100 with saline, in varying quantities from 1.0 to 6 ml. Each test was performed with three different concentrations of serum. In the majority of tests, amounts of 1.5, 2.0, and 2.5 ml. of diluted serum were satisfactory. After incubation at 37° C. for 40 minutes with frequent shaking, the tubes were centrifuged and the density of the supernates read in the Coleman Universal Spectrophotometer at 5500 Å. The density of the experimental tube divided by the density of a completely lysed control tube (approximately .560) gave the degree of hemolysis. The amount of complement was then determined by interpolation on the curve for the equation $x = (y/1 - y)^{1/n}$, where x = the amount of complement (C') in 50 per cent hemolytic units, y = the degree of lysis (7, 10). Preliminary tests with human complement showed that the factor $1/n$ equalled 0.2 as it had in the determination of guinea pig complement (8). Usually two, and occasionally all three, tubes containing the various amounts of test serum gave an amount of hemolysis between 10 and 80 per cent, and could be used for interpolation of the 50 per cent unit of complement. Not only was this a control of the tech-

nic at two or three different concentrations of serum, but it also permitted the detection of anticomplementary activity in the serum when increasing amounts of serum consistently showed proportionately less C' activity. This situation did not occur in the series of sera reported here.

*Error of technic:*³ Repeated determination of the same samples of serum at different times gave values to within ± 1.08 units of C' (± 1 standard deviation) when the same concentration of serum was used in the repeated tests. The results of determinations at two different degrees of hemolysis (between 5 per cent and 80 per cent), *e.g.*, with different amounts of the same serum, gave values of ± 1.8 units, as did the results of determinations at three different degrees of hemolysis in the same hemolytic range. Since the test point employed was close to the range of 50 per cent hemolysis, the standard deviation of ± 1.1 units is applicable to the determinations reported.

RESULTS

The mean value for the C' content of 50 normal individuals aged five to 39 was 37.7 ± 3.9 50 per cent hemolytic units per milliliter of serum. The distribution of the values in the normal subjects is

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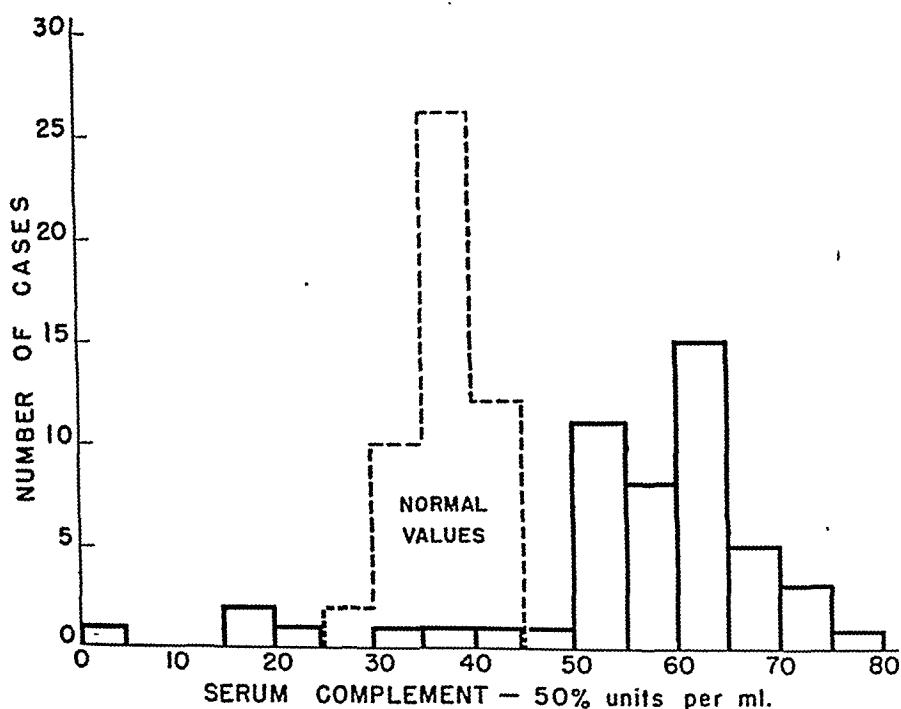


FIG. 1. DISTRIBUTION OF THE SERUM COMPLEMENT OF 50 NORMAL INDIVIDUALS AND INITIAL LEVELS OF 50 PATIENTS WITH ACUTE RHEUMATIC FEVER

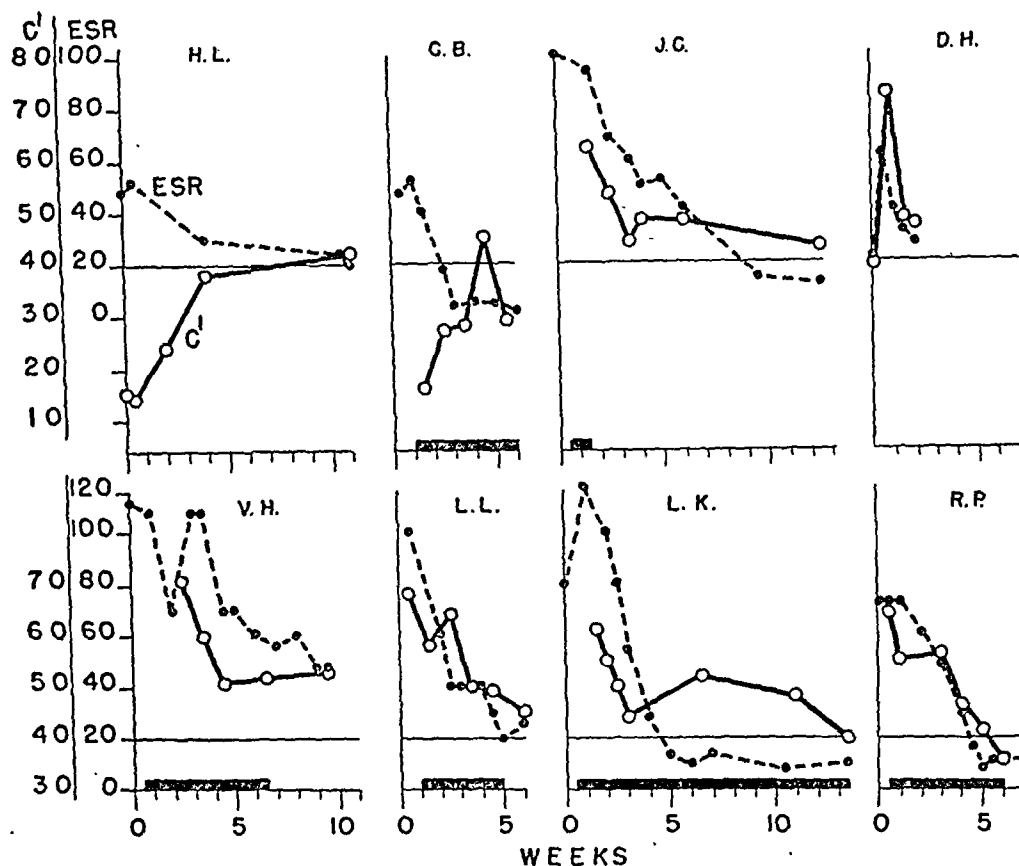


FIG. 2. REPRESENTATIVE CURVES OF THE C' CONTENT DURING THE COURSE OF ACUTE RHEUMATIC FEVER

Complement content in 50 per cent hemolytic units per milliliter of serum is charted by the open circles. The Westergren sedimentation rate (ESR) is charted by the closed circles and broken line. Heavy black lines indicate period of salicylate administration.

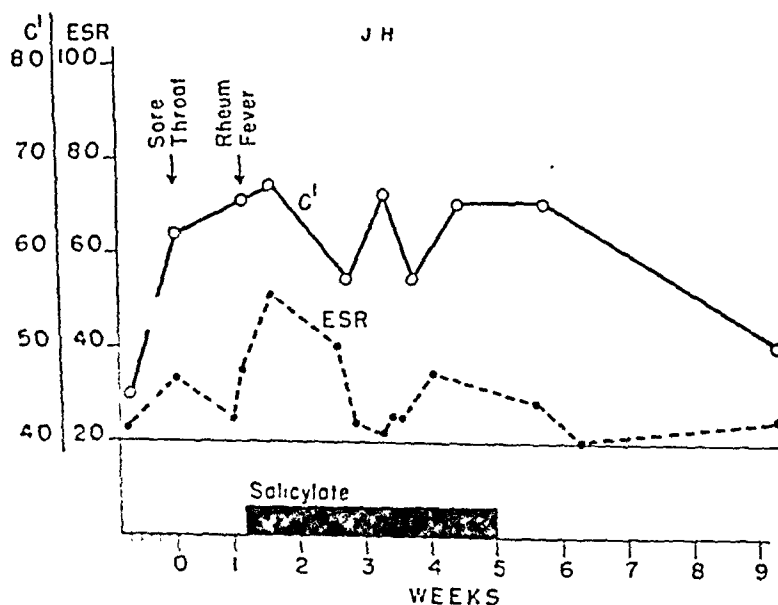


FIG. 3. SERUM COMPLEMENT LEVELS BEFORE AND DURING AN ATTACK OF ACUTE RHEUMATIC FEVER

represented by the dotted bar graph in Figure 1. The C' content of the sera of normal individuals was maintained over a period of months with little variation.

Serial determinations of C' were done on the sera from 50 patients with rheumatic fever. The initial values of complement in these individuals irrespective of the duration of the active disease before coming under observation are shown in Figure 1. These values, and the subsequent determinations of C' during the course of the illness, are presented in Table I. At the time of the initial determination, the serum complement was in excess of 50 units and well above normal in 42 of the 50 patients; three others, with initially normal levels, had a subsequent elevation. Twenty-four patients were seen within two weeks after the onset of symptoms of rheumatic fever (Tables IA and B) and 20 of these had C' levels in excess of 50 units per ml. Of the four patients seen in the first two weeks in which the level was below 50 units, one (D. H.) had a normal level on the day of onset (39 units). In this individual, an abrupt increase in C' was observed four days later concomitantly with the development of more manifest symptoms and an elevation of the erythro-

cyte sedimentation rate. Another of the four patients seen early in the course of the disease with a C' below 50 units died shortly after a single determination revealed no C' activity. The other two patients with initial low levels (H. L. and C. B. in Table IB and Figure 2) presented low curves which gradually reached normal with the subsidence of activity. These two patients did not present any other unusual finding or differ clinically from the majority of patients having high complement levels. There was no evidence of an accompanying acute nephritis, as might be anticipated from the observation that several cases of acute glomerulonephritis presented low curves (11). These two patients, therefore, are the only ones which presented low C' curves. The complement curves of six other individuals are presented in Figure 2 and are representative of the curves of the 40 patients with elevated complement levels. It is apparent that complement is high initially and falls gradually to normal. This fall accompanies the subsidence of activity, as indicated roughly by the curve of the erythrocyte sedimentation rates (millimeters after one hour, Westergren method). Although many patients developed a rise in anti-streptolysin "O" titers, there was no correlation

TABLE IA

Total serum complement in rheumatic fever related to the stage of the disease
Cases seen before and during acute exacerbation of rheumatic fever

Patient	Complement before the attack 50 per cent units per ml. serum		Complement after the attack 50 per cent units				Comments
			Week of disease				
			Onset	2nd	3rd	etc.	
J. H.	1 mo. before sore throat Sore throat 1 wk. before the attack	43 62	64	67	57, 66	57, 65, 65..50	Charted in Figure 3
A. N. G.	1 wk. before recrudescence	52	51	48	26	30, 38, 50, 45	Biphasic curve
A. S.	4 wks. before recrudescence	39	60	42	43		Charted in Figure 4
H. G.	2 mos. after subsidence of clinical activity. (ESR* 14)	50	60†	70†			Died of rheumatic myocarditis 2 mos. after last determina- tion
A. A.	2 mos. before recrudescence	36 42	53	48	64	62, 60, 44, 50	
J. M.	6 wks. before recrudescence 4 wks. before recrudescence 2 wks. before recrudescence	37 52 48	60		41	41 after 1 mo.	Charted in Figure 4

* Westergren sedimentation rate.

† Asymptomatic but ESR suddenly 100.

TABLE IB
Cases seen within two weeks of onset of acute rheumatic fever

Patient	Duration of illness before observation	Complement content of serum 50 per cent units per ml.					Comment
		Weeks under observation					
		1	2	3	4	etc.	
D. H.	1 day	39	76		48		C' paralleled rise and fall of ESR
A. Ek.	1 day	76		66			
V. Jam.	1 day	56, 68	67	75		57 43	
V. Ro.	2 days	50		42		35	
M. P.	2 days	70	72	60	53	45 45	Developed chronic activity for 4 mos. with C' of 49 to 53, then 40
T. W.	2 days	62	71				
H. L.	3 days	15, 14		24		38 42	Low curve
C. B.	5 days	17	27		28	45 29	Developed chorea Low curve at onset and later
L. L.	5 days	67	58	64	49	49 45	Mild thyrotoxicosis after third week
G. Je.	5 days	63	75	66	53	50 50	Continued activity
L. F.	1 wk.	63	66	63	59	48	
L. K.	1 wk.	61	55	50	44	52 48 40	
R. Ag.	1 wk.	55			47		
A. See	1 wk.	62		50			
J. Cam.	1 wk.	62	53	44	48	48 43	
N. Fa.	2 wks.	0					Died 3 days later; acute pulmonary edema
F. Aco.	2 wks.	56	52	80	70	80	Discharged active
J. Bar.	2 wks.	63	50	55		40 46	

with the rise and fall of serum complement content.

Six patients have been observed with complement determinations before a recrudescence of rheumatic activity (Table IA and Figures 3 and 4). An elevation of serum C' frequently preceded the onset or exacerbation of rheumatic activity either as a sequence of a sore throat, or as a manifestation of continuing subclinical rheumatic activity. The change in complement activity from the relatively normal state, with the onset of a hemolytic streptococcus infection and, subsequently, of rheumatic fever is seen in Figure 3. The patient, J. H., was being observed routinely in the follow-up clinic seven years after an attack of rheumatic fever and had a mild upper respiratory

infection due to an undetermined organism. Two months later, the patient experienced a streptococcus sore throat. The sedimentation rate rose, as did the complement level. With the occurrence of acute rheumatic fever ten days later, the serum complement remained elevated, with renewed elevation of the sedimentation rate. Observations to be reported show that many acute inflammatory illnesses such as acute pharyngitis are accompanied by an increase of serum complement (11). This increase abruptly subsides except in the few instances where active infection persists or, as in this instance, rheumatic fever develops. The character of C' levels in recurrent rheumatic attacks is also illustrated in the chart of three patients presented in Figure 4. These

children had had activity three to six months before the initial *C'* determinations. Despite the apparent subsidence of activity by the usual clinical and laboratory criteria at least three months previously, the first two children, J. M. and A. S., were kept on salicylates. Salicylate was then discontinued with recurrent manifestations of activity in both children. In the first patient, the *C'* was elevated before cessation of the salicylate and, in retrospect, can be considered an indication of continuing subclinical rheumatic activity. In the second individual a normal *C'* was present at the

time salicylate was discontinued but was elevated four weeks later. In the subsequent recrudescence, if such it may be called, no joint flare-ups, fever, or tachycardia accompanied the rise in sedimentation rate and *C'*. The third individual, H. G., had had an acute attack of rheumatic fever, which subsided promptly. The first observation of complement was done after discharge from the hospital and was somewhat elevated but the sedimentation rate was normal. After two months the ESR and *C'* were observed to be elevated and a tachycardia developed. No other manifestations were ob-

TABLE IC
Cases of rheumatic fever seen two or more weeks after onset

Patient	Duration of illness before observation	Weeks under observation					Comment
		1	2	3	4	etc.	
V. H.	3 wks.	71	60	51	52	53 in 15th wk.	Developed thyrotoxicosis
R. P.	3 wks.	65	55		56, 46, 41	35 in 9th wk.	
S. Cr.	3 wks.	41	53	38			Subsiding activity
R. Mc.	3 wks.	22					Died 6 wks. later
L. Go.	3 wks.	56	57	56	49, 49	50	
M. E.	3 wks.	64		50			
M. Do.	3 wks.	50	48	43		44	
J. Cul.	3 wks.	52	46	30			Died in 4th week
D. Pa.	4 wks.	66		50	50	32 in 10th wk.	
M. So.	4 wks.	61	63	53	54		Discharged active
M. Con.	4 wks.	52	42		39 inactive	39	
B. W.	4 wks.	65	49	56	49	59	Discharged active
A. K.	4 wks.	55	55		29	42 after 2 mos.	
V. Ack.	5 wks.	48	50	49	56	43	
T. Tho.	5 wks.	50		45	50	52	Continues active
A. Ada.	5 wks.	53					
C. Bre.	5 wks.	51	53, 47	37	39	33	
H. Am.	2 mos.	32		29		5 mos. later 37 (inactive)	
J. A. M.	5 mos.	73				37 quiescent 3 mos. later	
R. Mad.	6 mos. Low-grade activity	51	54				

TABLE ID

Chronically active rheumatic fever

Patient	Duration of illness before observation	Weeks under observation					Comment
		1	2	3	4	etc.	
M. Me.	2 mos.	64	52	57	41	56 74	Rise with reactivation .
V. Be.	3 mos.	60					
B. Ma.	8 mos.	65			55	54	Recurrent nodules, elevated, sed. rate
McR.	8 mos.	55			53		
N. Cl.	18 mos.	58	58				
A. Ei.	4 yrs.	51	53	46	54	54 after 4 mos.	

served at that time. Subsequently, a fulminating attack of acute rheumatic myocarditis developed which terminated with death two months later. It is probable that the initial observation of an elevated C' in this instance indicated continued sub-clinical activity, later becoming manifested by an elevated sedimentation rate as well, and then tachycardia, fever and frank myocarditis.

The presence of subclinical activity was also detected in the patient, A. A., of Table IA. This patient had had a rheumatic attack with prompt subsidence of activity two months previous to the first observation. The sedimentation rate was normal as was the complement content of the serum. Without any clinical changes, and with a continued normal sedimentation rate, the C' rose

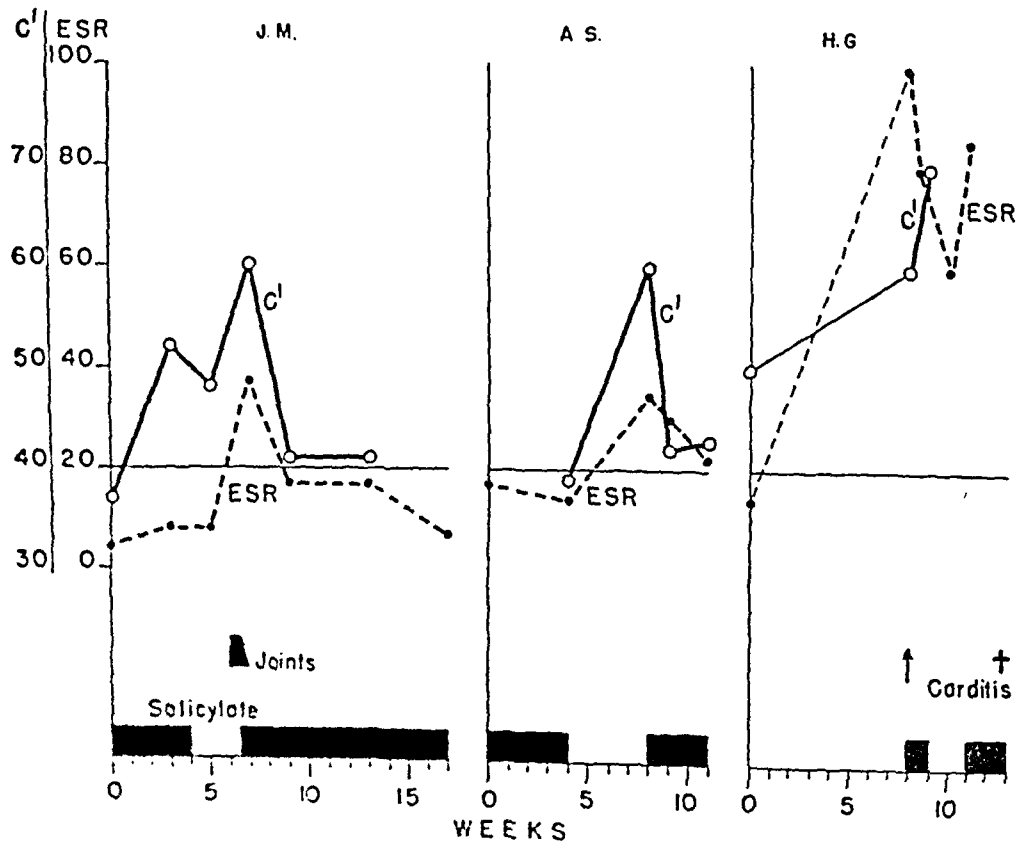


FIG. 4. THE COMPLEMENT AND SEDIMENTATION RATE OF THREE PATIENTS WITH RECURRENT RHEUMATIC FEVER WHO HAD HAD SUBSIDENCE OF CLINICAL ACTIVITY THREE TO SIX MONTHS PREVIOUSLY

after one month to 53 units and later to 64. After four weeks of unexplained elevation of the serum complement a moderate but persistent tachycardia was observed and an abrupt elevation of the sedimentation rate occurred.

DISCUSSION

Serum complement has been of interest to many investigators who attempted to define the rheumatic state as an allergic type of reaction (1-3, 12). A diminution of circulating complement is found in certain experimental antigen-antibody reactions, both *in vitro* (13) and *in vivo* (14). Clinically, a diminution of serum complement has been reported in some cases of serum sickness (11, 15, 16). However, there are several causes for a low C' other than fixation by antigen-antibody aggregates. Decreased production of C' is said to occur in liver disease (17), and in terminal illnesses (18). Also, C' levels apparently may be diminished by the presence of inhibitory or anticomplementary substances in the serum. Anticomplementary sera are occasionally found in routine Wassermann tests, but the nature of the substances responsible for the inhibition or inactivation of complement is not clear. One such substance is gamma globulin, a relative preponderance of which causes serum to become anticomplementary (19). This study, employing a precise and reproducible technic, failed to confirm the reports of a low complement in 46 of 50 cases of rheumatic fever. Of four cases with a low C' content, two died shortly after the determination. The observation is in agreement with previous reports (17, 18) of a low C' preceding death in many conditions other than rheumatic fever. Two patients with rheumatic fever exhibited low complement curves (Figure 2, H. L. and C. B.). No evidence of an abnormal anticomplementary effect was found in the sera of these patients to account for the low C' activity but the diminution may have been due to an antigen-antibody reaction that occurred independently of the rheumatic process.

It is known that certain antigen-antibody reactions do not fix complement *in vitro* (20). In addition, in several known allergic reactions, with clinical manifestations similar to serum sickness, but caused by a simple chemical antigen, *e.g.*, sulfadiazine, a high complement level was found (11). Because these immunologic or allergic reactions

may occur without any apparent fixation or diminution in complement, the possibility remains that the rheumatic reaction may be allergic and yet fail to fix complement. It is therefore apparent that the allergic hypothesis for the development of rheumatic fever receives neither support nor refutation by the data presented, although evidence for the occurrence of an antigen specific for rheumatic fever has not been substantiated (21).

This study fails to confirm reports of a low or normal C' in patients with rheumatic fever. Indeed, an unexpected finding in the opposite direction was observed. An increase in C' above 50 units per milliliter was observed at some time in 45 of the 50 cases observed. The increase was particularly marked early in the course of the disease. Very few reports in the literature mention an increase in C' in acute inflammatory disorders (6, 22), possibly because the customary use of double dilution of serum (*viz.*, .01, .02, .04 ml. with a normal value of 0.02 ml.), does not permit increased C' activity to be noted except where the increase is 100 per cent or more above normal. In addition, maximal C' activity may have been prevented by the lack of optimal concentrations of Mg^{++} and Ca^{++} (8). Using the technic of Mayer *et al.*, a high C' was also found in a variety of other acute and chronic illnesses (11). The cause for the increase in C' activity is not apparent. Although the complement response in rheumatic fever does not appear to be specific for this disease, the mechanism of this increase warrants further study, as it may be concerned with resistance mechanisms. Serum complement is a complex of labile substances which apparently plays a part in resistance mechanisms of the host. It is instrumental in promoting phagocytosis of bacteria and probably has a wide range of more ill-defined activities (17, 23-25). The increase in C' in inflammatory conditions resembles somewhat other non-specific changes in the blood such as the elevation of the sedimentation rate and the appearance of the C-reactive protein of Tillett and Francis (26) (the acute phase protein).

The elevation of complement in most cases of rheumatic fever is related to the course of the disease. It may remain abnormally elevated with apparent subsidence of activity and the return to normal of other criteria such as the polymorphonuclear leukocyte count or the ESR. In these in-

stances it appears to be a more sensitive indication of continuing activity than the ESR. However, it is not an infallible aid because it, as well as the sedimentation rate, may occasionally be normal a few days before obvious clinical exacerbation occurs. Furthermore, the extent of increase in complement does not appear to be related to the severity of the disease process but may perhaps be related to the abruptness of the change from the normal to the pathological state. Further studies concerning the relationship of an increased C' content of serum to activity of the rheumatic process are in progress.

SUMMARY

1. Quantitative serial studies of total complement (C') in serum were made, using the spectrophotometric technic for the determination of the 50 per cent hemolytic unit in the presence of magnesium and calcium ions.

2. The range of complement in sera of 50 normal individuals was found to be 37.7 ± 3.9 units per milliliter.

3. Forty-five of 50 patients with rheumatic fever had an elevation of the serum complement above 50 units per milliliter of serum at the onset of the attack or shortly thereafter. Two patients presented a low complement which gradually became normal. Of the remaining patients, two others had a low C' and died, and one patient had a normal C' level determined when the disease process was beginning to subside.

4. The elevation of serum complement appears to be another criterion of activity of the rheumatic process.

5. The relationship of these observations to the hypothesis that the rheumatic state is of allergic origin is discussed.

ACKNOWLEDGMENTS

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EVALUATION OF THERAPEUTIC SUBSTANCES EMPLOYED FOR THE RELIEF OF BRONCHOSPASM. V. ADRENERGIC AGENTS¹

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Many laboratory techniques have been employed in the study of sympathomimetic amines, particularly in an attempt to correlate chemical structure with pharmacological activity (1-7). In Table I are shown the structural formulae of the preparations dealt with in this report.

The technique of protection studies in asthmatic human subjects appears to offer an additional method of investigating and evaluating these and other substances in the ultimate test object, man. The technique essentially consists of the measurement of the protection afforded by any substance against the dyspnea and bronchospasm induced by repeated administrations of histamine, methacholine, or allergens, as measured, in part at least, by changes in vital capacity.

METHODS

The methods we have employed and their historical development have been extensively presented elsewhere (8, 9).

In the experiments reported at this time, the bronchospastic agents used were histamine diphosphate⁶ and methacholine chloride,⁷ employed intravenously or as aerosols. The protecting ability of the therapeutic agent under examination was determined by serial observations of the reduction in vital capacity produced by repeated administration of a constant dose (predetermined as capable of producing a consistent reduction in vital capacity

of at least 25 per cent) of histamine or methacholine at varying intervals after the protecting agent was administered. The degree of protection may be described in terms of the following equation:

$$P = \frac{C - E}{C} \times 100,$$

where P is the degree of protection in per cent, C the control drop in vital capacity produced by administration of the bronchospastic substance (before the protecting drug is given), and E the drop similarly produced at any time after the protecting agent has been administered. These percentage values may then be massed into averages derived from several experiments on different asthmatic subjects. Determination of such averages is necessary, for we have repeatedly encountered single protection studies, or even repeated studies in the same individual, which were at variance with the average of results obtained by employing the same drugs in many experiments carried out on many individuals.

RESULTS

1. *Epinephrine hydrochloride.* Epinephrine hydrochloride 1:1000, 0.5 cc. subcutaneously, cause the usual side-reactions of throbbing in the chest and head, uneasiness, tremor, etc. Protection was striking, however, in that this dose afforded 100 per cent "immediate" (five to ten minutes after injection) protection against intravenous histamine. This decreased gradually reaching a level of 40 per cent after 143 minutes (Figure 1). This 40 per cent level has previously (9) been proposed as the minimum at which protection may be considered significant, in view of the errors inherent in any technique of clinical assay. Against the bronchospastic effects of methacholine, epinephrine hydrochloride 1:1000, 0.5 cc. subcutaneously, provided 90 per cent immediate protection and maintained a significant level, over 40 per cent, for 83 minutes. These results are presented in tabular form in Table II.

Subcutaneously administered epinephrine was also assayed against the bronchospastic effect of aerosols of histamine and methacholine and dis-

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⁶ Kindly supplied by Abbott Laboratories, Inc., Chicago, Illinois.

⁷ Kindly supplied by Merck and Company, Rahway, New Jersey.

TABLE I

Structural relationships of the sympathomimetic agents studied. (Modified from Gunn [3])

						
NEOSYNEPHRIN	H	OH	H	OH	H	CH ₃
EPINEPHRINE	OH	OH	H	OH	H	CH ₃
VAPONEFRIN	OH	OH	H	OH	H	CH ₃
ISUPREL	OH	OH	H	OH	H	HC(CH ₃) ₂
EPHEDRINE	H	H	H	OH	CH ₃	CH ₃
ORTHOXINE	H	H	OCH ₃	H	CH ₃	CH ₃

Vaponefrin is a specially prepared racemic epinephrine.

played somewhat less, though still very effective protecting ability. Against histamine it showed 70 per cent immediate protection, which rose to a peak of 86 per cent at 40 minutes, and maintained a level greater than 40 per cent for 127 minutes. Less protection against methacholine was again observed; there was an immediate level of 40 per cent, rising to a peak of 51 per cent at 38 minutes, and remaining at a significant level for 72 minutes.

The 1 : 100 dilution of epinephrine hydrochloride (10) was dispensed in a glycerine-water solution, as were all other agents employed as aerosolized mists. Deep inhalations at ten-second intervals

were administered with a standard Vaponefrin hand-bulb nebulizer until six were completed. Against intravenous histamine 75 per cent immediate protection was observed. The significant level of 40 per cent lasted for 28 minutes. Against intravenous methacholine there was 56 per cent immediate protection and an 18 minute duration of the 40 per cent level (Figure 2).

The protective capacity of epinephrine aerosol appeared slightly greater when assayed against nebulized bronchospastic agents. There was 89 per cent immediate protection and 36-minute duration of the 40 per cent level against histamine;

EPINEPHRINE HCL 1:1000
0.5 CC. S.C.

--- HISTAMINE IV (7 TRIALS)
— MECHOLYL IV (7 TRIALS)
--- HISTAMINE AER (4 TRIALS)
— MECHOLYL AER (4 TRIALS)

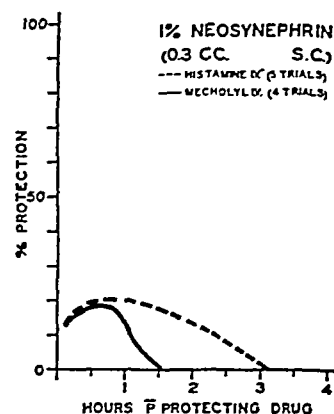
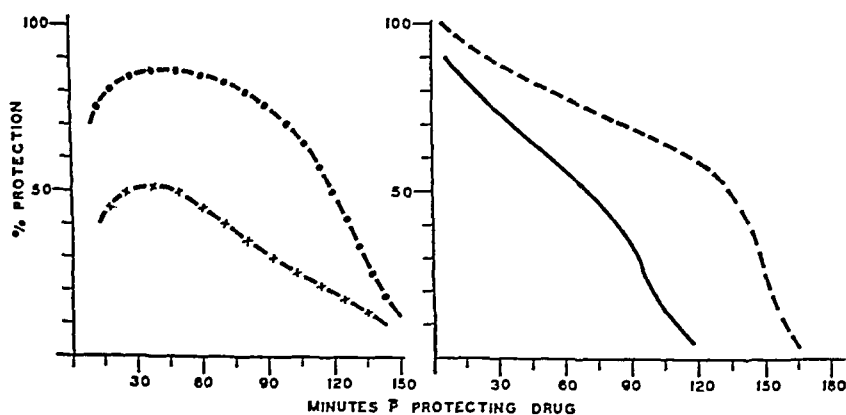


FIG. 1. THE PROTECTION AFFORDED AGAINST HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL) BY 0.5 CC. OF EPINEPHRINE HYDROCHLORIDE 1:1000, AND 0.3 CC. OF 1 PER CENT NEOSYNEPHRIN ADMINISTERED SUBCUTANEOUSLY

TABLE II

Summary of the pertinent data obtained with the phenylethylamines when tested against the bronchospastic effects of histamine and methacholine (referred to below as mecholyl)

BRONCHOCONSTRICTING AGENT

PROTECTING DRUG	HISTAMINE IV		MECHOLYL IV.		HISTAMINE AER.		MECHOLYL AER.	
	IMMEDIATE PROTECTION (%)	DURATION OF SIGNIFICANT (40%) PROTECTION (MINUTES)	IMMEDIATE PROTECTION (%)	DURATION OF SIGNIFICANT (40%) PROTECTION (MINUTES)	IMMEDIATE PROTECTION (%)	DURATION OF SIGNIFICANT (40%) PROTECTION (MINUTES)	IMMEDIATE PROTECTION (%)	DURATION OF SIGNIFICANT (40%) PROTECTION (MINUTES)
EPINEPHRINE 1:1000, 0.5CC., SC	100	143	90	83	70	127	40	72
EPINEPHRINE 1:100 AER.	75	28	56	18	89	36	66	17
VAPONEFRIN 2.25 % AER.	93	27	74	18	90	53	87	40
NEOSYNEPHRIN 1:100, 0.3CC., SC	13	0	13	0				
NEOSYNEPHRIN 1:100 AER.	52	16	32	0				
ISUPREL 1:100 AER.	98	62	91	81				
ISUPREL 1:200 AER.	70	22	69	42	84	21	65	25
ISUPREL 1:5000, 0.5CC., SC	100	30	56	18				

and 66 per cent immediate and 17-minute duration of the 40 per cent level against methacholine. These data are not, however, significantly different from those obtained with the intravenous bronchospastics.

2. *Vaponefrin*.⁸ This is a 2.25 per cent solution of a specially prepared synthetic racemic epinephrine. Richards, Barach and Cromwell (11) considered it preferable to the official 1:100 solution of epinephrine hydrochloride, which is the levo-rotatory isomer. We have compared these aerosols elsewhere (12).

Using histamine intravenously we found that six deep inhalations of Vaponefrin aerosol provided 93 per cent immediate protection and significant protection (over 40 per cent) lasting 27 minutes. Against intravenous methacholine we obtained an immediate level of 74 per cent and a duration of a significant level of 18 minutes. Employing the bronchospastics by nebulization resulted in no greatly significant difference in intensity of action, 90 per cent against histamine and 87 per cent against methacholine. However, the 40 per cent level was maintained definitely longer, 53 minutes with histamine and 40 minutes with methacholine (Figure 2). The explanation for this discrepancy is obscure; it may simply be due to inadequate sampling.

3. *Neosynephrin*. As may be seen from Table I, this drug differs from epinephrine only in the absence of the para-hydroxyl group on the aromatic portion of the molecule. The compounds resulting from removal of one or both hydroxyl groups are more stable but have greatly decreased inhibitory effects. Various investigators (11, 13-15) have found neosynephrin of little value as a bronchodilator although it may be an efficient bronchovasoconstrictor (14, 15).

Utilizing the 1:100 solution of neosynephrin, we have studied the protecting effect of the drug administered by nebulizer and by hypodermic injection. Our studies were limited to intravenous use of the bronchospastic agents. Figure 1 portrays the ineffectiveness of 0.3 cc. subcutaneously, the level of protection never exceeding 20 per cent. Neosynephrin aerosol, 1:100, in the usual dose of six deep inhalations, proved somewhat more effective (Figure 2), displaying against histamine 52 per cent immediate protection which dropped below 40 per cent within 16 minutes. Against methacholine no significant protective levels were attained, 32 per cent being the highest observed.

4. *Isuprel*.⁹ This sympathomimetic amine, 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol differs from epinephrine only in the alkyl group

⁸ Kindly supplied by the Vaponefrin Co., Upper Darby, Pennsylvania.

⁹ Kindly supplied by Winthrop-Stearns, Inc., New York, New York.

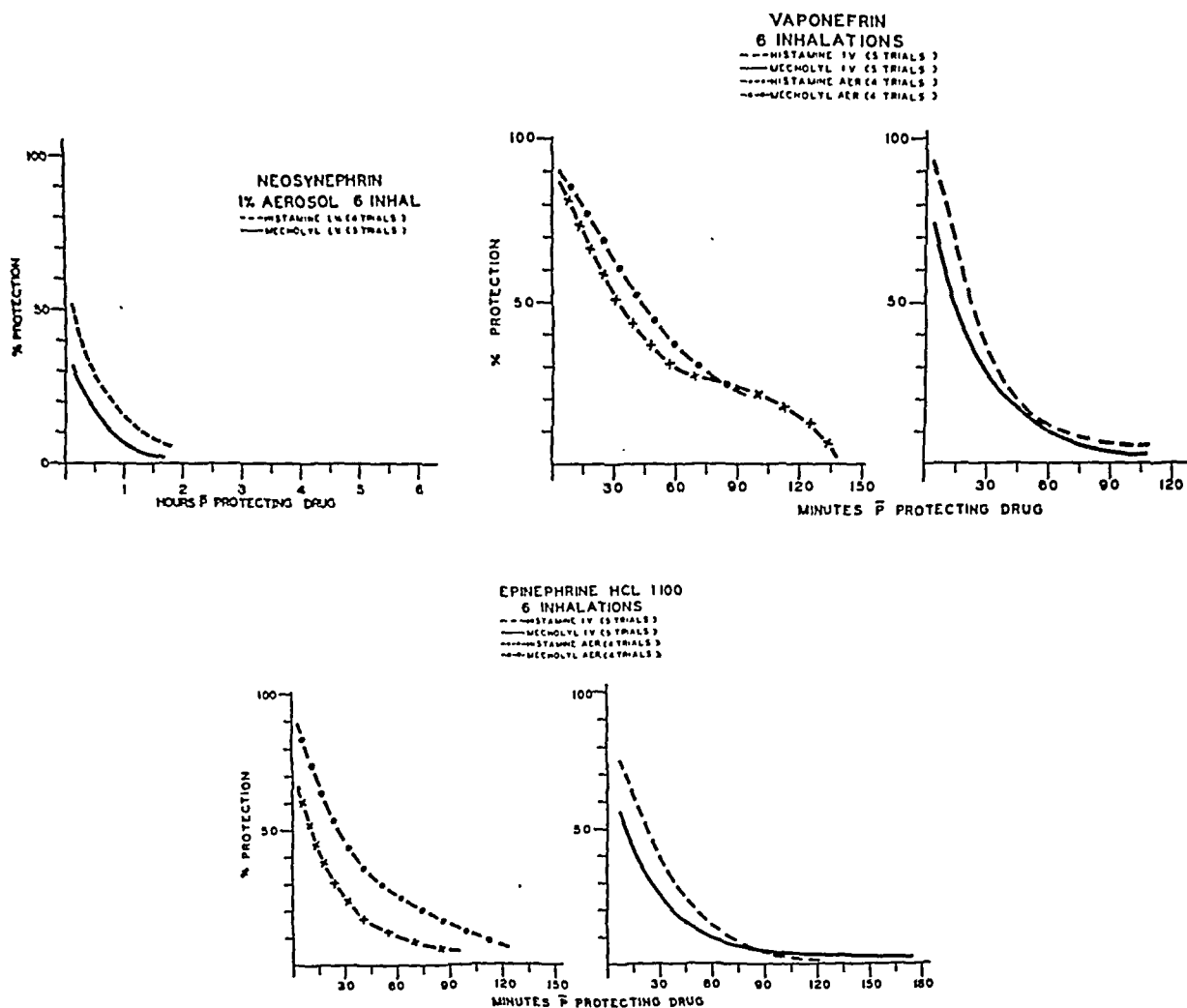


FIG. 2. THE PROTECTION NOTED AGAINST HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL) BY NEBULIZED SOLUTIONS OF EPINEPHRINE HYDROCHLORIDE (1:100), VAPONEFRIN (2.25 PER CENT) AND NEOSYNEPHRIN (1 PER CENT)

attached to the nitrogen atom (Table I). It has been demonstrated that substitution on the amino nitrogen of branched alkyl groups, particularly isopropyl (as in Isuprel) and *tert*butyl, produces compounds with enhanced sympathin I-mimetic properties (4-7, 16). The specific structure of the substituted alkyl group is also important in influencing the inhibitory potency of such compounds.

The clinical efficacy of Isuprel has been previously reviewed by Segal and Beakey (17, 18), who introduced its use in this country. Cohen and Van Bergen (19) have found Isuprel a most effective antagonist of methacholine-induced bronchospasm in animals and of spontaneous asthma in man.

We have employed a 1:50,000 dilution of Isuprel intravenously in a few subjects with variable results. Occasionally the most dramatic possible complete relief of clinical asthma has been noted, while a dose of 0.25 cc. (0.005 mgm.) was still being administered. The effect persisted for a matter of hours. In other instances relief was only partial and transient after intravenous administration of 0.25 cc. (0.005 mgm.) to 0.5 cc. (0.01 mgm.), even in repeated doses. The 0.25 cc. dose caused a uniform increase of 20-30 beats per minute in the heart rate. All patients experienced unpleasant, although not alarming, palpitations. The 0.5 cc. dose, on the other hand, accelerated the pulse by 50 beats per minute and caused uneasiness and alarming palpitations. The

effect on blood pressure was minimal. The dose of 0.005 mgm. appeared to cause a small increase in systolic pressure (approximately 10 mm. of mercury), probably due mainly to the increased rate. There appeared to be, in general, a tendency toward a slight fall in the diastolic level. The larger dose, 0.01 mgm. (0.5 cc.), caused a transient drop in systolic and diastolic levels simultaneous with the greatest increase in heart rate. We performed several protection studies using this very dilute solution of Isuprel intravenously. Doses of 0.25 cc. (0.005 mgm.) and 0.5 cc. (0.01 mgm.) failed to demonstrate any protective ability against histamine or methacholine for long enough periods to be measured by the method employed.

Isuprel 1:100, the dilution commonly employed

with epinephrine, provided essentially complete immediate protection against intravenous histamine and methacholine (98 and 91 per cent respectively). Not only did it maintain a significant (40 per cent) protection against histamine for 62 minutes, but it also afforded a surprising 81-minute duration against methacholine (Figure 3). However, this dilution of Isuprel frequently caused unpleasant epinephrine-like side-effects.

Isuprel 1:200 is the dilution in common use for nebulization. It is, of course, one-half as concentrated as the usual epinephrine solution and equivalent to approximately one-third the concentration of levo-epinephrine in Vaponefrin. This dilution of Isuprel displayed (Figure 3) 70 per cent immediate protection against intravenous histamine with a duration of 22 minutes for

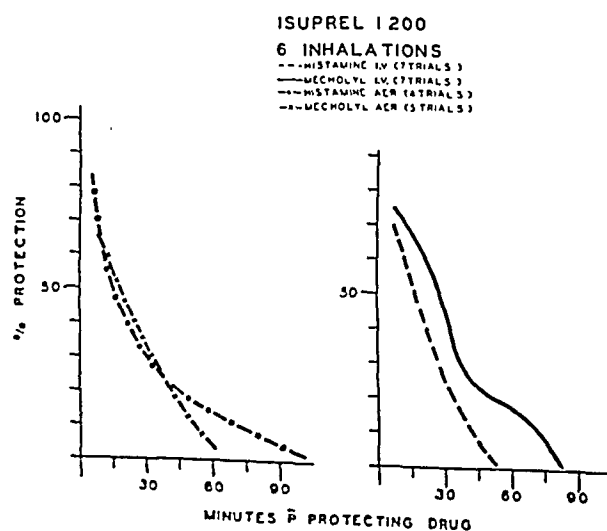
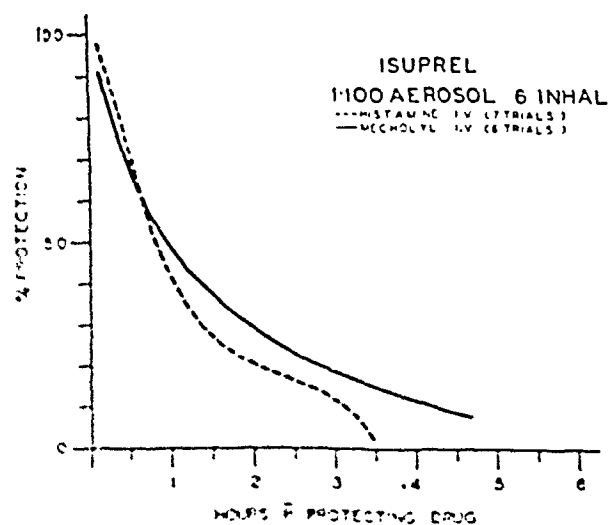
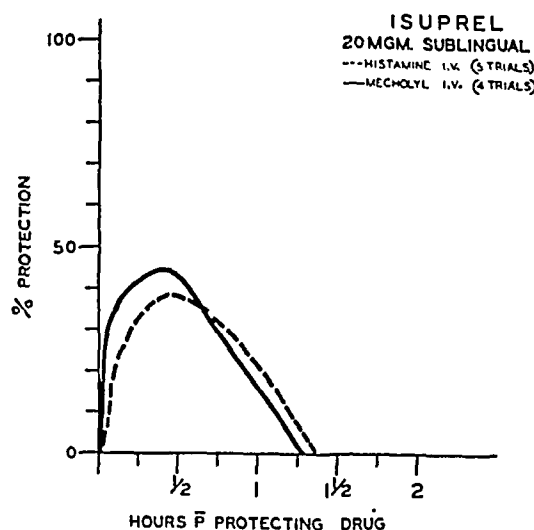
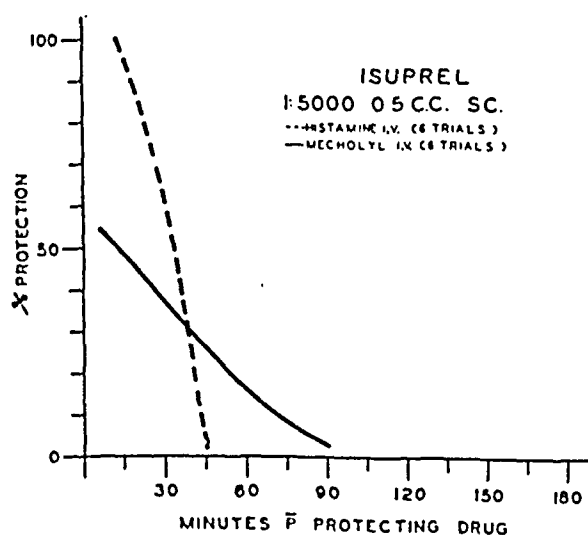


FIG. 3. THE PROTECTIVE CAPACITY OF ISUPREL ADMINISTERED BY VARIED ROUTES AND IN VARIED AMOUNTS AGAINST HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL)

significant levels. Against intravenous methacholine, there was a 69 per cent immediate level and significant levels persisted for 42 minutes. As may be seen in Table II, the data were, with one exception, essentially equivalent when the bronchospastics were employed in the form of aerosols. An immediate level of 84 per cent and a 21-minute duration of significant protection were found against histamine; and 65 per cent with 25 minutes were the comparable values for methacholine. The only discrepancy appears in the duration of levels over 40 per cent against methacholine administered intravenously and as an aerosol. This, again, may be due to inadequate sampling and the consequent chance inclusion of patients with extreme differences in responsiveness to Isuprel.

An average dose of the 1:5000 dilution of Isuprel for subcutaneous injection is 0.5 cc. This dose was assayed against intravenous histamine and methacholine (Figure 3). We obtained complete (100 per cent) immediate protection against histamine and a continuation of significant levels for 30 minutes. Less anticholinergic effect was noted with this preparation, for the initial intensity was only 56 per cent, and it dropped to 40 per cent in 18 minutes.

Sublingual tablets of Isuprel, although they have been moderately effective clinically in occasional patients, have not proved to be an adequate substitute for a nebulizer in most cases. We have performed protection studies with these tablets in doses of 20 mgm. (two tablets) against histamine and methacholine administered intravenously. Against histamine, significant levels of protection were not attained, and a peak of 38 per cent was reached in 27 minutes. Against methacholine the results were very similar although the 40 per cent level was reached in 12 minutes after the tablets dissolved under the tongue. (Time for dissolution was usually two to three minutes.) The total duration of levels over 40 per cent was 23 minutes. However, the peak level, attained 24 minutes after dissolution of the tablets, was only 44 per cent. These data are presented graphically in Figure 3.

5. *Ephedrine*. From Table I it may be seen that ephedrine is lacking in the hydroxyl groups on the benzene ring, and possesses an additional methyl group attached to the alpha-carbon atom.

Clinically it has longer, less intense action than epinephrine; it is active orally.

In our studies, employing the usual oral dose of 25 mgm. (administered several hours after the subject's last meal), there was a delay of 81 minutes before a protection level of 40 per cent was reached against histamine (Figure 4). A peak level of 57 per cent was attained at 120 minutes. Significant levels of 40 per cent or over were maintained for 108 minutes, or until slightly over three hours after the drug was taken. Against methacholine, no significant levels of protection were reached. The peak level was only 32 per cent, occurring 60 minutes after ingestion of the therapeutic agent.

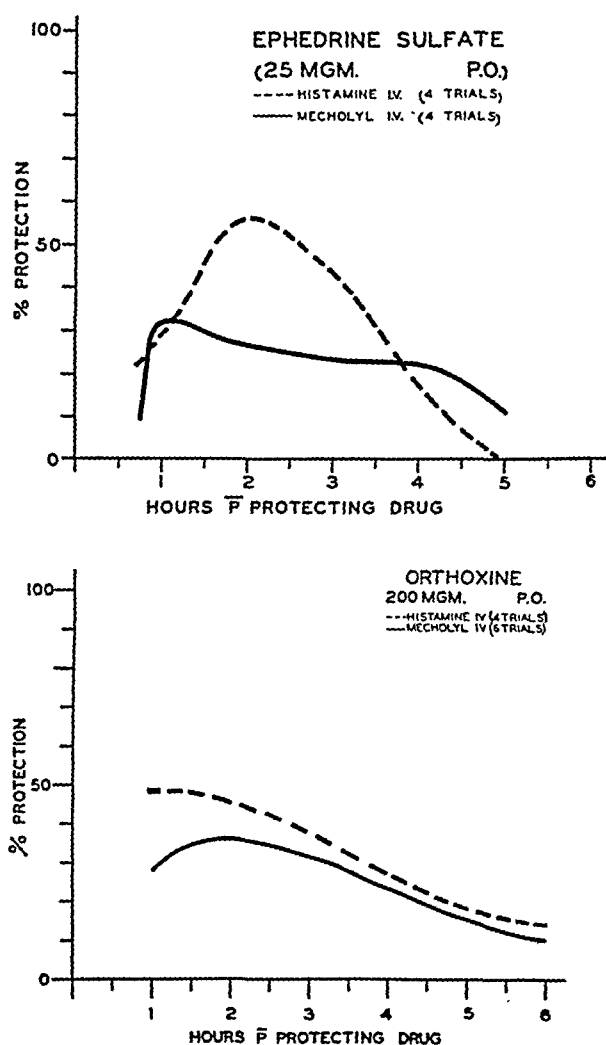


FIG. 4. THE PROTECTING ABILITY OF ORALLY ADMINISTERED EPHEDRINE SULFATE (25 MGM.) AND ORTHOXINE (200 MGM.) AGAINST HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL)

6. *Orthoxine*.¹⁰ This synthetic amine differs from ephedrine in the absence of the beta-hydroxyl group and the presence of a methoxy radical in the ortho position.

We have made a clinical trial in approximately 20 patients with a dose of 100-200 mgm. three to four times daily. In general it proved to be of little benefit in preventing severe asthmatic attacks, although one patient obtained moderate relief and one noted marked improvement. Side-effects were observed by many patients and varied among anorexia, nausea, light-headedness, dizziness and drowsiness. Only one patient complained of the ephedrine-like effect of insomnia. The syrup of orthoxine has been tolerated fairly well and appears to give clinical relief from troublesome cough in some patients.

In protection studies against intravenous histamine, orthoxine, in a dose of 200 mgm. orally, was apparently rapidly absorbed, for it displayed 49 per cent protection at 57 minutes. In addition, this proved to be the peak level attained inasmuch as the protective capacity dropped very gradually to 40 per cent at 165 minutes after ingestion of the drug. The total measured duration of significant protection was 108 minutes. Against methacholine, a 40 per cent level was never reached. The peak attained was 38 per cent at 120 minutes. These figures for orthoxine, graphically presented in Figure 4, obviously are very closely similar to those for ephedrine.

SUMMARY AND CONCLUSIONS

1. Five well-known sympathomimetic amines and one new one have been studied with respect to their protective capacity against the bronchospastic effects of histamine and methacholine in sensitive asthmatic patients.

2. No consistent difference in results was observed whether the bronchospastic agents were administered intravenously or as aerosols.

3. Epinephrine hydrochloride 1:1000, 0.5 cc. subcutaneously, protected as well as or better than the other agents tested and afforded greater duration of significant protection. However, its cardiovascular and central nervous stimulating side-reactions proved disagreeable.

¹⁰ *Orthoxine* supplied by The Upjohn Co., Kalamazoo, Michigan.

4. Isuprel 1:100 aerosol (double the usual strength), six inhalations, was comparable to the subcutaneously administered epinephrine, particularly in anticholinergic effect, although it protected less than half as long against histamine. This, too, caused undesirable side-effects.

5. Of the three most commonly employed solutions for nebulization, Vaponefrin, 2.25 per cent, appeared to have a slightly greater antihistaminic protecting ability than Isuprel 1:200 and epinephrine 1:100. The latter two were of approximately equal efficacy as antihistaminics. Isuprel 1:200 demonstrates greater anticholinergic protecting ability than either of the other two preparations.

6. Neosynephrin was quite ineffective when injected parenterally and of only slight value by the inhalatory route.

7. Sublingually administered Isuprel, in a dose of 20 mgm., proved to have little protective capacity against the bronchospastic effects of histamine and methacholine. It was occasionally of moderate value clinically when administered by this route.

8. Isuprel 1:5000, 0.5 cc. subcutaneously, displayed excellent though brief protecting ability against the bronchospastic effects of histamine and considerably less effectiveness against methacholine.

9. Isuprel 1:50,000 could not, because of cardiovascular side-effects, be injected intravenously in doses sufficient to effect measurable protection. Doses of 0.25 cc. and 0.5 cc. (0.005 to 0.01 mgm.) occasionally proved dramatically effective in alleviating clinical asthma, although it usually was less effective and quite transiently so.

10. A new sympathomimetic amine, orthomethoxy-beta-phenylisopropyl methylamine hydrochloride (*Orthoxine*, *Upjohn*), of the class of ephedrine, was studied and appeared quite similar to the latter in protecting capacity. Brief clinical trial indicated that the ortho-methoxy group may occasionally reverse the central stimulation of ephedrine.

ACKNOWLEDGMENT

We are indebted to Dr. H. J. Rubitsky for carrying out several protection study tests in this series.

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EVALUATION OF THERAPEUTIC SUBSTANCES EMPLOYED FOR THE RELIEF OF BRONCHOSPASM. VI. AMINOPHYLLINE¹

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The use of various xanthine derivatives in the management of bronchial asthma was first described by Herrmann and Aynesworth (1) in 1937. They were able to demonstrate definite increases in the vital capacity of patients with bronchial asthma after the intravenous administration of aminophylline. The clinical contributions of Barach (2), Cooke (3), Unger (4), Segal (5) and others amply describe the valuable role played by this medication in the management of patients seriously ill with bronchial asthma.

We have previously described in detail (6-10) the historical development of a technique for evaluation of medications employed in the treatment of bronchial asthma by testing the efficacy of such compounds in preventing the decrease in vital capacity artificially induced in sensitive asthmatic subjects by the administration of suitable bronchospastic agents (histamine, methacholine, or allergens). The exact relationship of the dyspnea and bronchospasm induced by histamine or by methacholine to that occurring in spontaneous bronchial asthma is uncertain. However, since it is probable that only asthmatic or potentially asthmatic individuals respond to these agents by changes in vital capacity (7, 11, 12), this technique of assay in man may be of value. This report will concern itself with the results obtained with theophylline-ethylene diamine (Aminophylline, U.S.P.).

METHODS

A protection study consists of the observation of the decreases in vital capacity produced by the repeated ad-

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ministration of a bronchospastic agent (by any of various routes) before and after a protecting drug is administered. A "control" drop, which should be equal to at least one-quarter of the resting vital capacity, is first established; this drop should preferably exceed 1000 cc. The protecting agent is then administered (by any of various routes) and bronchospasm re-induced at suitable intervals until the effect of the protecting agent disappears. We have devised an equation (1, 2) by which the degree of protection afforded by any given drug at any time may be expressed in percentage:

$$P = \frac{C - E}{C} \times 100,$$

where P represents the degree of protection in percentage, C equals the control drop in vital capacity before the protecting drug is given, and E is the drop produced at any time after the protecting drug has been administered. These data, being independent of vital capacity determinations, may be grouped into averages. Since we have repeatedly encountered individual protection studies which vary greatly from the mean derived from a group of studies, we have computed all our data on the basis of such averages derived from at least four individual protection studies on different subjects.

RESULTS

By means of this technique we have measured the protecting ability of aminophylline, administered by various routes, against the decrease in vital capacity induced by the intravenous or aerosol administration of histamine or of methacholine.

(1) *Intravenous aminophylline vs. intravenous histamine and methacholine.* After the intravenous injection of 0.5 Gm. of aminophylline in 20 cc. of distilled water, over a period of ten minutes, protection against the bronchospastic effects of histamine⁶ and of methacholine⁷ was observed immediately. Against histamine, this immediate level was 65 per cent; against methacholine it was only 37 per cent. In view of the many unavoidable

⁶ The preparation used (histamine diphosphate) was kindly supplied by Abbott Laboratories Inc., North Chicago, Illinois.

⁷ The preparation used ("mecholyt chloride") was kindly supplied by Merck and Co., Rahway, New Jersey.

errors inherent in any technique of clinical assay, we have considered all protection values below 40 per cent as insignificant. With this figure as a standard, it is apparent that intravenous amino-

phylline displays a protecting action against methacholine which is just short of being significant. Significant protection against intravenous histamine persisted for two hours and ten minutes

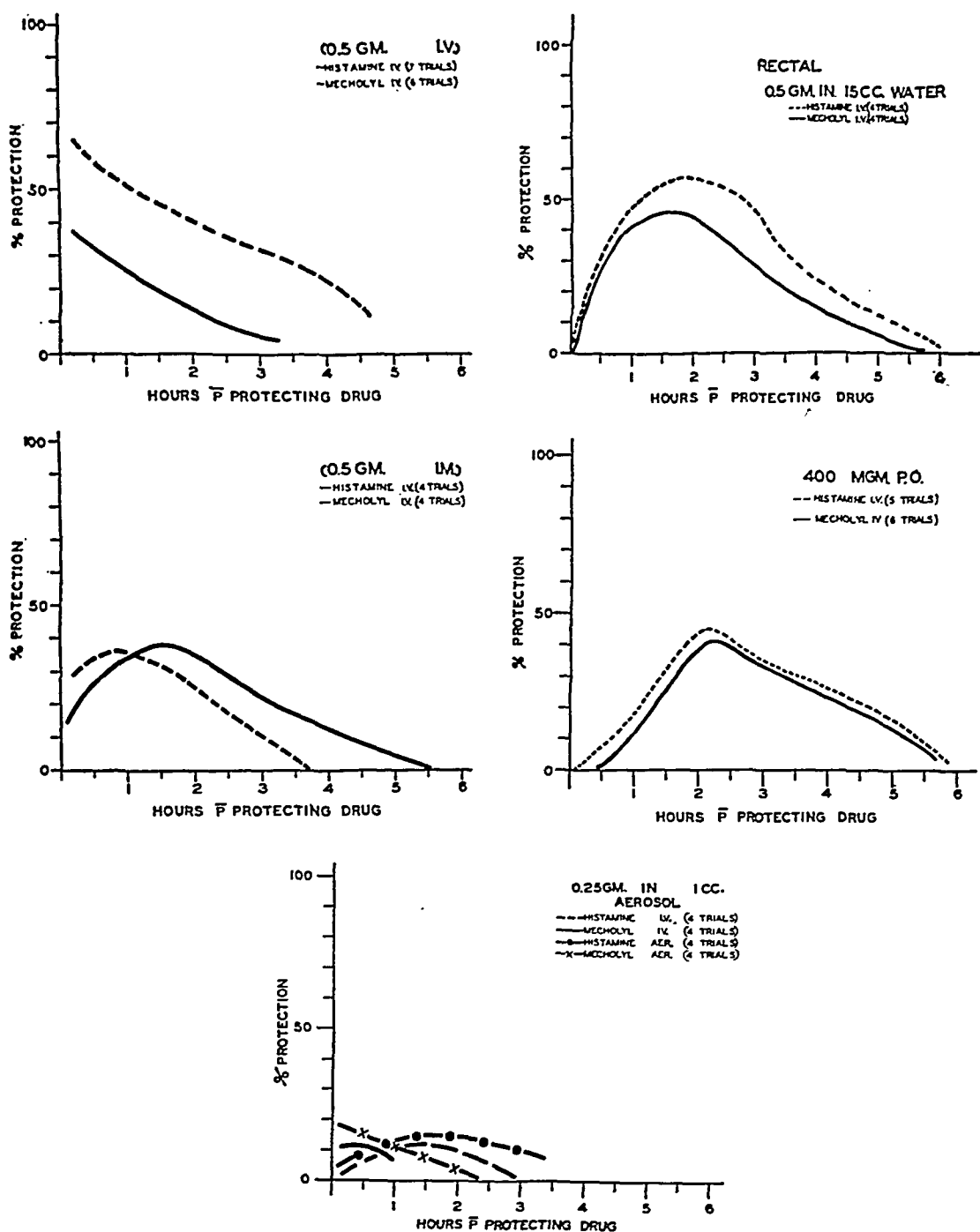


FIG. 1. THE PROTECTING ACTION OF AMINOPHYLLINE, ADMINISTERED BY VARIOUS ROUTES, AGAINST THE DYSPNEA AND BRONCHOSPASM PRODUCED BY HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL)

TABLE I

The relative intensity and duration of the protecting action afforded by aminophylline administered by various routes against the effects of histamine and methacholine (referred to below as mecholyl)

BRONCHOCONSTRICTING AGENT

PROTECTING DRUG	HISTAMINE IV.				MECHOLYL IV.			
	LATENT PERIOD OF DEVELOPMENT OF 40% LEVEL (MIN.)	PEAK LEVEL %	PEAK TIME P INJ. (MIN.)	DURATION OF SIGNIFICANT 40% PROTECTION (MIN.)	LATENT PERIOD OF DEVELOPMENT OF 40% LEVEL (MIN.)	PEAK LEVEL %	PEAK TIME P INJ. (MIN.)	DURATION OF SIGNIFICANT 40% PROTECTION (MIN.)
AMINOPHYLLIN 0.5 GM. IN 20 CC. IV.	0	65	IMMED.	130	—	37	IMMED.	0
AMINOPHYLLIN 0.5 GM. IN 2 CC. IM.	—	36	50	0	—	38	90	0
AMINOPHYLLIN 0.5 GM. IN 20 CC. H ₂ O RECTALLY	45	58	120	150	55	45	105	90
AMINOPHYLLIN 0.4 GM. (CT) ORALLY	105	45	130	50	130	42	135	20
AMINOPHYLLIN 0.25 GM. IN 1 CC. AEROSOL	—	12	75	0	—	12	15	0

COMPARATIVE PROTECTIVE VALUE OF AMINOPHYLLIN ADMINISTERED BY VARIOUS ROUTES AGAINST HISTAMINE AND MECHOLYL-INDUCED DYSPNEA AND BRONCHOSPASM IN ASTHMATIC SUBJECTS.

* AMINOPHYLLIN AEROSOL ALSO EXHIBITED NO SIGNIFICANT PROTECTION AGAINST THE EFFECTS OF HISTAMINE AND MECHOLYL AEROSOLS

(Figure 1, Table I). The lesser degree of protection displayed against the effects of intravenous methacholine was of shorter duration as well.

(2) *Intramuscular aminophylline vs. intravenous histamine and methacholine.* Administration of 0.5 Gm. of aminophylline in 2 cc. of distilled water intramuscularly in the gluteal region led to protection against the decrease in vital capacity induced by the intravenous administration of histamine and methacholine which never quite achieved significant levels (Figure 1, Table I). In this case, as in the case of intravenous aminophylline and methacholine, the maximum protecting levels were just below our arbitrary level of significance, being 36 per cent in the case of intramuscular aminophylline against histamine, and 38 per cent against methacholine. These peak levels were reached after a considerable delay (50 minutes for histamine, 90 minutes for methacholine) and were maintained for comparatively brief intervals.

(3) *Rectal solution of aminophylline vs. histamine and methacholine.* A solution of aminophylline containing 0.5 Gm. in 15 cc. of distilled water was instilled rectally by means of a Dakin syringe and a well-lubricated No. 12 Fr. urethral catheter. Aminophylline so administered resulted in significant protection (above 40 per cent) against the bronchospastic effects of intravenous histamine for two and one-half hours and against intravenous methacholine for one and one-half hours. This occurred after delays of 45 and 55 minutes respectively, presumably incident to absorption. After the rectal administration of 0.5

Gm. of aminophylline, a peak level of 58 per cent protection against histamine was attained in two hours; 45 per cent protection against methacholine was attained in one and three-quarters hours (Figure 1 and Table I).

(4) *Oral aminophylline vs. intravenous histamine and methacholine.* Orally administered aminophylline in the form of two compressed tablets of 0.2 Gm. each yielded protection to a lesser degree against the decrease in vital capacity produced by subsequently administered intravenous histamine and methacholine. Barely significant peak values of 45 and 42 per cent against histamine and methacholine respectively were obtained (Figure 1 and Table I). Significant protection (40 per cent) was attained only after a delay of one and three-quarters hours in the case of histamine, and two hours and ten minutes in the case of methacholine. With histamine, the duration of significant protection was only 50 minutes; with methacholine, 20 minutes.

(5) *Aerosol aminophylline vs. aerosol and intravenous histamine and methacholine.* The relative ease of aerosol administration of small quantities of concentrated solutions of adrenergic agents has led us to investigate the properties of an aerosol produced with the standard Vaponefrin nebulizer from a 25 per cent solution of aminophylline. The disagreeable taste of this preparation may be partially masked by the addition of a drop or two of spirits of peppermint. We first investigated the protecting action of six inhalations of such a mist, produced with hand-bulb nebulization, but

were unable to demonstrate any protecting action against the bronchospastic effects of subsequently administered histamine or methacholine, the latter being given both intravenously and as an aerosol. We then nebulized 0.25 Gm. of aminophylline in 1.0 cc. of distilled water employing the Vaponefrin nebulizer with oxygen flows, using the intermittent Y-tube occlusion technique. This procedure is time-consuming and the aerosol is irritating to the pharynx and trachea. No significant protection could be demonstrated (Figure 1 and Table I). Peak protection values in all instances reached only 12 to 17 per cent.

(6) *Direct comparison of various routes.* The striking disparity in protection obtained after the administration of aminophylline via various routes led us to compare the antihistaminic potency of aminophylline in each of the five routes employed. In two subjects, R. L. and J. S., aminophylline was administered on successive visits to the laboratory via each of the five routes employed, so that a more direct comparison between these methods of administration might be made (Figure 2). It is apparent from these curves that in both cases optimum antihistaminic activity appeared after the rectal administration of a solution containing 0.5 Gm. of aminophylline. In these two individuals intravenous and intramuscular administration were of essentially equal activity; the intravenous route, however, has the advantage of immediate

action. Orally administered aminophylline in a slightly smaller dose was definitely less effective than intravenous or intramuscular medication. Aerosol aminophylline was, as previously noted, totally ineffective.

DISCUSSION

In contrast to the data obtained by similar examination of the protecting ability of various adrenergic agents (10) against the decrease in vital capacity produced by intravenous injections of histamine and methacholine, it is apparent that aminophylline must exert its bronchospasmolytic action in a different manner. Such anti-asthmatic drugs as the sympathomimetic amines have a rapid, intense protecting action in minute doses, whereas the effect of aminophylline is milder and more prolonged and the dose employed is many times greater. In dosage and in the time sequence of its protecting action, aminophylline approaches more closely the antihistaminic drugs (blocking agents), data on which will be presented elsewhere (13).

The administration of aminophylline solution by rectum has been extensively popularized in recent years (2). We have long been aware of the powerful bronchospasmolytic action of such medication in the management of patients with severe asthma. This impression is amply borne out by these studies, in which rectally administered

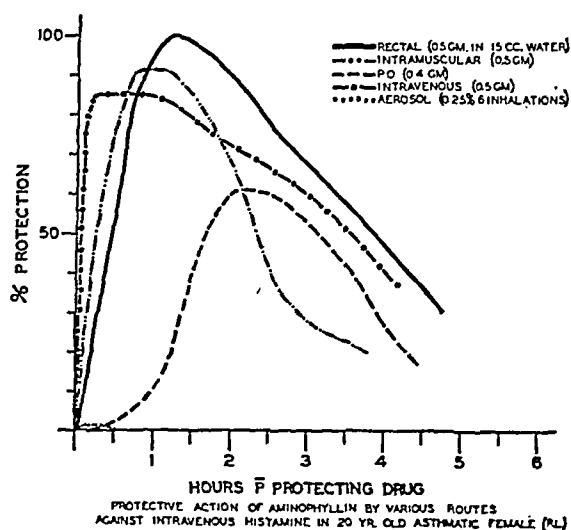
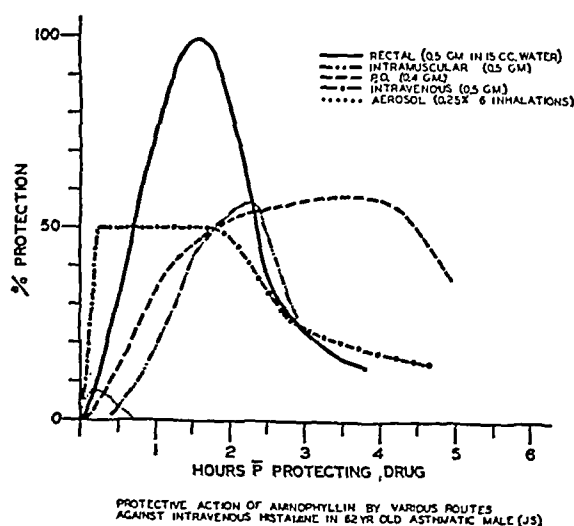


FIG. 2. THE COMPARATIVE PROTECTING ACTION OF AMINOPHYLLINE ADMINISTERED BY EACH OF FIVE ROUTES TO TWO ASTHMATIC PATIENTS AGAINST THE DYSPNEA AND BRONCHOSPASM PRODUCED BY HISTAMINE AND METHACHOLINE (REFERRED TO ABOVE AS MECHOLYL)

aminophylline exhibits a protecting action equal or superior to all other routes, if the 45 to 60 minute absorption delay time is not objectionable. As absorption after oral administration is slower and as the period of significant protection is shorter, this route can have only limited value. Intramuscular aminophylline made a poor showing, one not entirely unexpected in the light of clinical experience. Were the pain usually attendant upon such an injection not already a sufficient deterrent to its clinical use, the lack of significant protecting ability should discourage dependence upon this route. It is our feeling that rectally administered aminophylline solution affords the patient a potent means for relief of asthmatic bronchospasm; moreover, the ease of self-administration of the rectal solution is, of course, an even more important factor. Where maximal immediate effect is essential, the intravenous administration remains the route of choice.

SUMMARY

1. The protecting ability of aminophylline, administered by various routes, against the dyspnea and bronchospasm produced by the administration of histamine and methacholine by various routes is presented.

2. The intravenous administration of 0.5 Gm. of aminophylline results in immediate protection against the bronchospastic effects of histamine and methacholine; the protection against histamine, which is definitely of significant degree, persists for two hours and ten minutes. Protection against methacholine falls below an arbitrary limit of significance.

3. Rectal administration of a solution of 0.5 Gm. of aminophylline yields a degree of protection against the dyspnea and bronchospasm produced by intravenous histamine and methacholine equal or superior to that produced by intravenous injection of the same dose of the drug. There is a 45 to 60 minute delay incident to absorption from the rectal mucosa, whereas the effects of the drug are apparent immediately following intravenous injection.

4. Aminophylline given intramuscularly (0.5 Gm.) is relatively ineffective in counteracting the bronchospastic effects of intravenous histamine and methacholine.

5. The oral administration of 0.4 Gm. of aminophylline results in relatively low degrees of protection as compared to that obtained with the rectal route. Significant protection appears only after a long delay and persists for an extremely short period of time.

6. Aminophylline in the form of an aerosol mist is almost totally ineffective in preventing the dyspnea and bronchospasm produced by intravenous or aerosol administration of histamine or of methacholine.

ACKNOWLEDGMENT

We are indebted to Dr. H. J. Rubitsky for carrying out several protection study tests in this series.

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CHLORAMPHENICOL (CHLOROMYCETIN) IN THE TREATMENT OF TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS)

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The present paper augments information previously published on the first 25 patients with scrub typhus to receive chloromycetin (1) and reviews our experience in the use of this drug in the treatment of a total of 69 persons who contracted tsutsugamushi disease in the vicinity of Kuala Lumpur, Federation of Malaya. The results obtained in this group are compared with those observed in 19 patients with the disease who received only supportive therapy and in three who were given p-aminobenzoic acid.

MATERIALS AND METHODS

Chloromycetin. Chloromycetin¹ prepared by the fermentation process was supplied for the current work by Parke, Davis and Company. A small amount of the antibiotic, subsequently administered in capsules, was received as pure crystalline material but almost all of the drug was in the form of tablets containing 0.25 gm. amounts of active crystalline material. All treatment was by the oral route.

The levels of chloromycetin in the serum and other body fluids were determined by a modification (2) of the technique described by members of the Research Laboratories of Parke, Davis and Company (3). This is a turbidimetric bioassay method in which the amount of drug in a known standard required to produce 50 per cent inhibition of growth of *Shigella paradysenteriae* (Sonne)² is compared with the inhibiting capacity of varying dilutions of the unknown material.

Isolation of rickettsiae. The presence of rickettsemia in patients was demonstrated in the following manner. Freshly drawn blood was defibrinated, by shaking with glass beads, and centrifuged; the plasma was discarded and the sedimented red cells were resuspended in an equal volume of physiological saline solution. One-half cc. of such a suspension was injected intraperitoneally

into each of four normal young adult albino mice of one of several inbred lines. Mice inoculated with blood containing rickettsiae generally showed signs of illness between the ninth and 12th days, and died between the 11th and the 14th. With few exceptions, the rickettsial strains recovered in the vicinity of Kuala Lumpur were highly virulent for mice, and usually all of the animals which received the infected blood died unless sacrificed for study. One mouse from the original group was sacrificed when it first showed signs of illness or on the 12th day. When the results were positive, these animals displayed the typical gross pathological changes of experimental *Rickettsia tsutsugamushi* infection in mice, i.e., enlargement of lymph nodes and injection of vessels observed when the ventral skin was reflected, sero-gelatinous peritoneal exudate, enlargement of the spleen, and accumulations of pleural fluid. Impression smears from the surface of the spleen and scrapings from the peritoneal surface of the abdominal wall, when stained by Giemsa's method, contained intracellular rickettsia-like organisms. The presence of such macroscopic lesions and the finding of rickettsiae microscopically were considered presumptive evidence of *R. tsutsugamushi* infection. Almost without exception rickettsiae were demonstrated microscopically in at least two of the four mice originally injected with the patient's blood. Furthermore, in the majority of instances a splenic suspension of material, or peritoneal exudate, from a mouse of the original group was passed by intraperitoneal injection to a second group of four normal animals in which the disease picture was reproduced. On a number of occasions the agent was maintained for several months by serial passages in mice. The strain obtained from treated Patient 9 was used as challenge material to demonstrate the immunity of occasional mice convalescent from infections with other strains and to determine whether mice had had inapparent infection following inoculation with a patient's blood. Seven of the strains were brought to the Army Medical Department Research and Graduate School where they were shown by reciprocal cross-immunity tests to be related to the well known Karp strain of *R. tsutsugamushi*. It was our experience that the attempted isolation of rickettsiae from blood of patients generally gave clean-cut results in the original mice. Thus, the employment of the blind passage technique and the testing of surviving mice for immunity by challenge with known lethal amounts of rickettsiae

¹Chloramphenicol is now the generic name for the antibiotic originally designated chloromycetin. Chloromycetin has been reserved as a trade name by Parke, Davis and Company.

²This organism is now classified by Bergey (1948) as *Shigella sonnei*.

rarely yielded indications of rickettsemia in the patient unless the original mice had shown obvious disease. It may be noted that strains of *R. tsutsugamushi* found in different geographical areas vary considerably in their pathogenicity for mice (4). Therefore, it was fortuitous and fortunate that the Malayan strains were relatively easy to recover and identify.

Serological tests. Weil-Felix tests were performed using standardized suspensions of OX-K and OX-19 strains of *Bacillus proteus* according to the technique of the Army Medical Department Research and Graduate School (5) or that of the Institute for Medical Research. Specific complement-fixation tests employing the washed rickettsial antigens of *R. prowazeki* and *R. mooseri* (6) were done in selected instances. Similarly, agglutination tests for *Leptospira icterohemorrhagiae* were performed on specimens from certain patients by the Veterinary Division of the School.

Routine clinical laboratory procedures. Erythrocyte and leukocyte counts and differential white blood cell counts were made in the usual manner. Hemoglobin values were determined spectrophotometrically by the cyanomethemoglobin method (7) and the usual procedures were employed for urinalysis. Blood levels of para-aminobenzoic acid were determined by a colorimetric procedure (8).

RESULTS

Patients with scrub typhus acquired during normal occupational duties

Selection of patients. Thirty patients who acquired scrub typhus during normal occupational duties were treated with chloromycetin. Twenty-eight of these contracted their disease in the vicinity of Kuala Lumpur. Twelve of the group became infected while working at the Seaport Estate between Kuala Lumpur and Batu Tiga (see Figure 1). The air field on the outskirts of Kuala Lumpur provided four of the cases who were treated. The remaining 12 occurred singly or in small groups within a 20 mile radius of the Institute for Medical Research. Patient 9 contracted his infection in Singapore and Patient 49 acquired hers following an accident at the Army Medical Department Research and Graduate School while handling the strain from treated Patient 25.

The course of the disease in patients who were given only supportive treatment was recorded in 19 persons who were observed during the period of the study. All of these acquired their infection in the general area which supplied the majority of the patients who received chloromycetin and five of them became infected on Seaport Estate. These

untreated patients were observed during periods when a supply of the specific antibiotic was not available, or the drug was withheld because they were first seen late in the disease and were not then desperately ill.

No effort was made to restrict the administration of chloromycetin to alternate patients who came under study. A precise balance could not have been obtained in this relatively limited investigation by selecting alternate patients for treatment unless certain important factors were evenly distributed in the treated and control groups; a few of these factors were age, sex, race, history of previous exposure in infected areas, and the virulence of the infecting strain of *R. tsutsugamushi*. Nevertheless, many of these variables were eliminated in the group of 17 patients from Seaport Estate in which five served as untreated controls. Early in the work it became quite clear that the course of the disease in the treated patients was markedly different from that observed in untreated persons in the current group or in patients with scrub typhus who had been studied during the preceding 20 years by members of the Institute for Medical Research (9-11). This marked difference in response of treated and untreated patients greatly simplified the interpretation of results.

Observations on patients with scrub typhus given supportive treatment. Detailed data on the 19 cases of scrub typhus who did not receive specific therapy are given in Table I. The more pertinent information is summarized in Table II. As the tabular data indicate, the average duration of fever in this group of persons was 17.1 days with extremes of 12 and 31. The course of the disease was complicated in two of the 19 patients by pneumonia and one of them died as a result. A third patient had suppurative parotitis which required surgical intervention.

All of these untreated patients were diagnosed on the basis of the history, clinical findings, and a positive Weil-Felix reaction. The diagnosis was further established in untreated Patient 16 by recovery of *R. tsutsugamushi* from the patient's blood.

Observations on patients with scrub typhus treated with chloromycetin. Information on each of the 30 treated patients and a summary of certain of the data are given in Tables II and III, respectively. The response following administra-

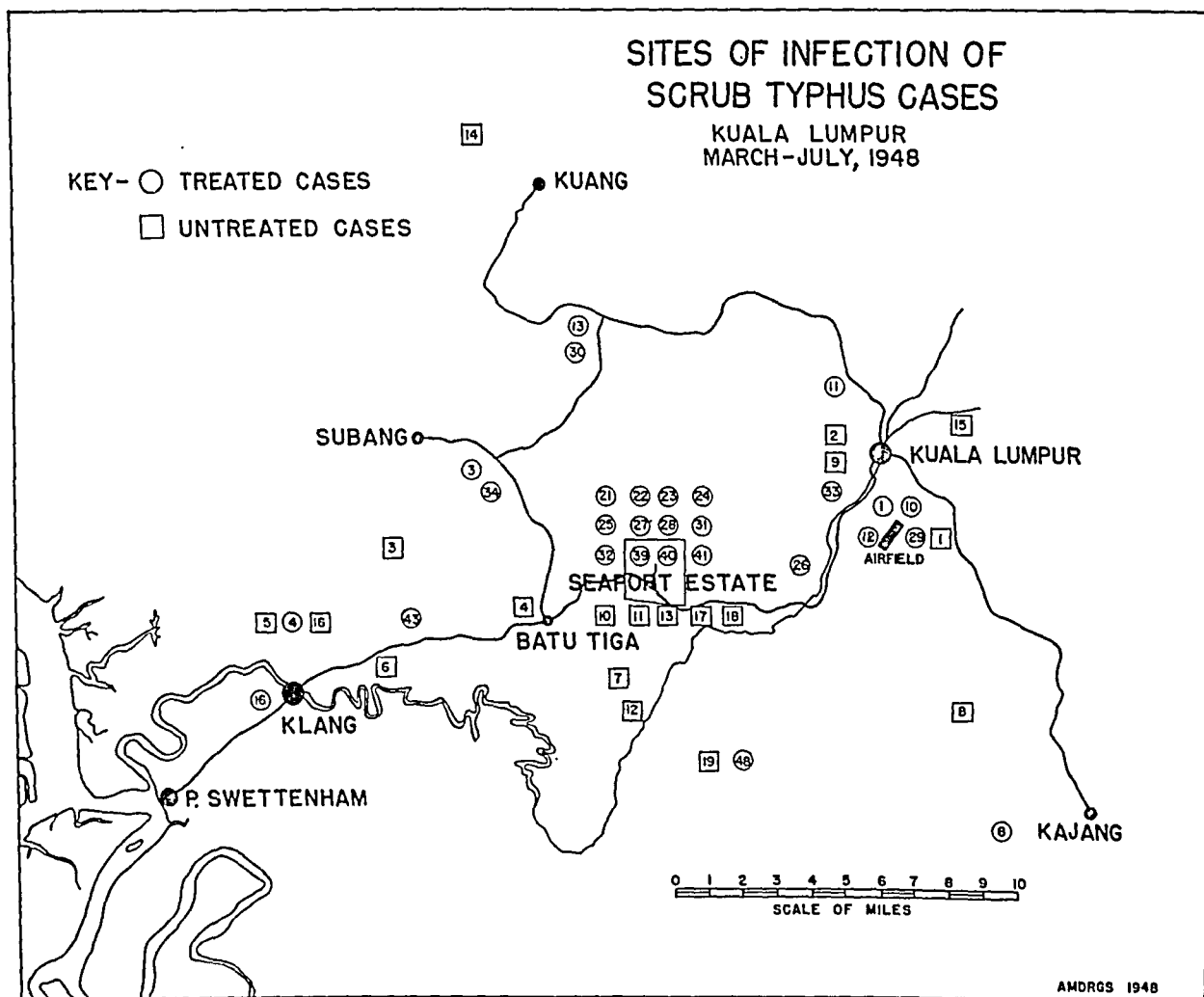


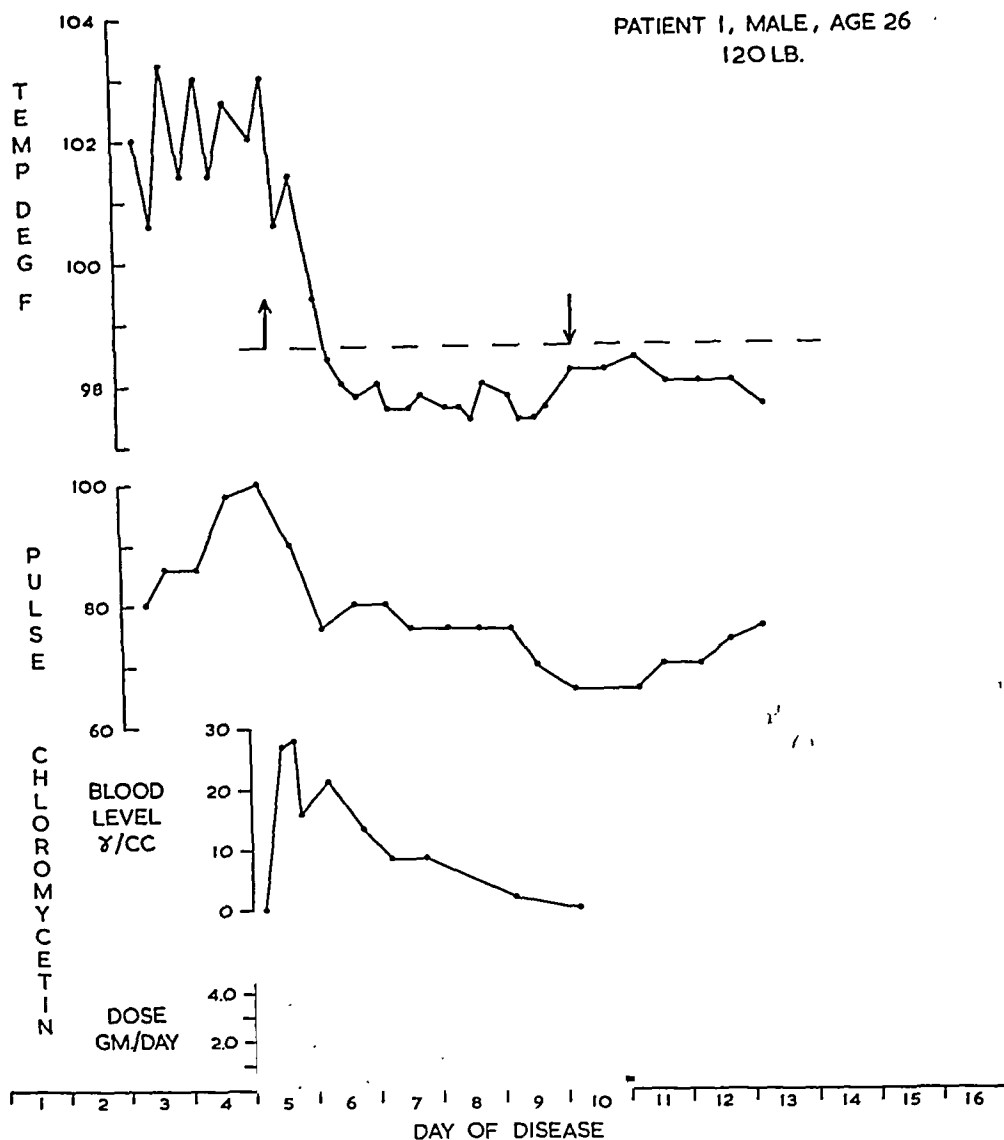
FIG. 1

tion of chloromycetin has been so consistent that it will be sufficient to present certain illustrative cases and to draw general conclusions.

All cases were given an initial oral dose of chloromycetin of about 60 mg./kg. of body weight. Since most of the patients were of small stature and weighed from 50–60 kg., this initial dose was usually between 3.0 and 3.5 gms. Following this dose, the patients were maintained for a variable period of time on 0.25 gm. amounts of drug administered at intervals of three hours. For example, treated Patient 1, whose clinical record is graphically presented in Figure 2, followed this regime for 48 hours and was then maintained on drug in reduced amounts for the three succeeding days. This patient who was started on chloromycetin on the fifth day became afebrile within 20 hours and remained free of fever thereafter. As therapeutic experience increased the total amounts of drug and the duration of therapy were

reduced (see Table III). Treated Patient 29, whose record is illustrated in Figure 3, was started on specific therapy on the sixth day and received a total of 6.5 gms. over a period of 22 hours. He became afebrile within 24 hours after the initial dose. A short course of drug such as used for Patient 29 provided results which appeared to be as satisfactory as those obtained when larger amounts were given over a period of four or five days.

The clinical diagnosis of scrub typhus was confirmed in each of the 30 patients by demonstration of rickettsemia or the development of a positive Weil-Felix reaction (see Table III). Rickettsiae were isolated from the bloods of 26 of the 30 patients. At least a four-fold increase in titer of agglutinins for the OX-K strain of *B. proteus* was shown to develop during convalescence in 28 of the patients. Convalescent sera from treated cases Nos. 12 and 24 had OX-K agglutinins in low titer



RASH	+++	++	+	0	0				
ESCHAR	+	+	+	+	+	+	±	0	
B.P.				104/70	100/70	98/72	104/70	96/70	104/70
WBC (THOUSANDS)							6.0		7.2
RBC (MILLIONS)							4.0		4.2
WF OX-K			80		320		1280		5120
RICKETTSSEMIA	+	+	+	0					

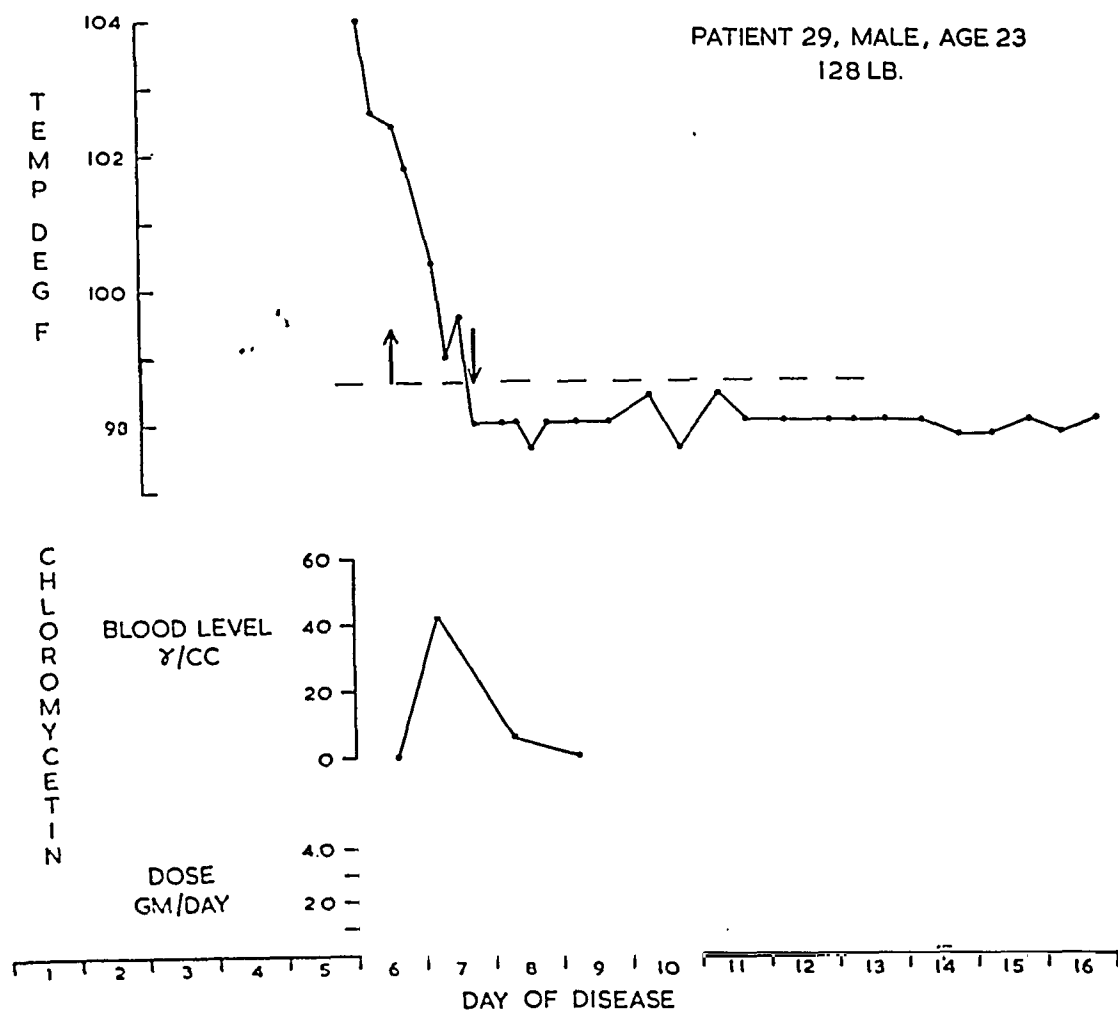
FIG. 2. CLINICAL RESPONSE OF PATIENT 1 TO CHLOROMYCETIN THERAPY BEGUN ON THE FIFTH DAY OF DISEASE

A total of 11.6 gms. of chloramphenicol was given over a period of 120 hours.

but did not contain a significant increase over the acute phase sample. The diagnosis was established, however, in each of these patients by the isolation of *R. tsutsugamushi*.

A comparison of the results obtained in the treated and untreated groups of patients is given in Table II. In the treated cases recovery began within a matter of hours after specific therapy was instituted, and fever lasted from six to 96 hours after chloromycetin was begun; the average duration being 31.8 hours for the entire group of 30 patients. It may be mentioned that treated Pa-

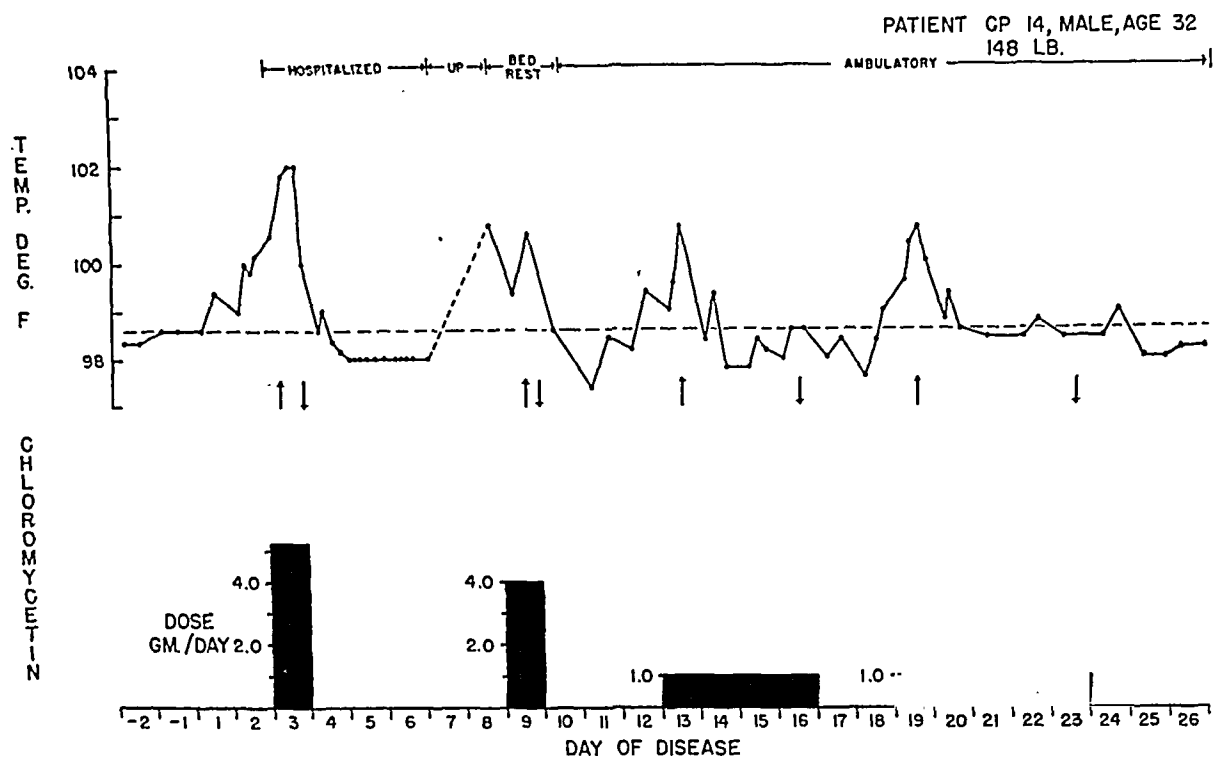
tient 9, who required 96 hours to become afebrile, was irrational, cyanotic and *in extremis* when treatment was started late on the ninth day. Although defervescence was slow this patient began to improve clinically within 12 hours after the antibiotic was administered, for at that time he sat up in bed and complained because breakfast was inadequate. It is worthy of note that chloromycetin therapy was equally effective whether given early or late in the disease. Thus, treated Patients 28 and 31, who were first seen on the third day, required 36 hours to become afebrile



RASH			++	+	0		
ESCHAR	+	+	+	+	±	0	
WF OX-K			80				320
ROCKETTSEMA		+	+	+	0		

FIG. 3. CLINICAL RESPONSE OF PATIENT 29 TO CHLOROMYCETIN THERAPY BEGUN ON THE SIXTH DAY OF DISEASE

A total of 6.5 gms. of chloramphenicol was given over a period of 22 hours.



RASH	+ ++ ++ + ± ± + + ± 0															
ESCHAR	+	+	+	+	+	+	+	+								
WF OX-K	40				40				1280							
RICKETTSEMI	+	+						+								

FIG. 4. CLINICAL RESPONSE OF PATIENT CP 14 TO CHLOROMYCETIN THERAPY BEGUN ON THE THIRD DAY OF DISEASE

He relapsed on three occasions, each recurrence responded promptly to additional treatment with chloromycetin.

while treated Patient 13, who was given the antibiotic on the 11th day, became afebrile within 24 hours. The average day on which therapy was begun for the treated group was 6.2 and the last febrile day of the illness was 7.4. This may be compared with that of the last febrile day of illness in the untreated group; this varied between 12 and 31 and averaged 17.1 days.

The first treated patient, although afebrile on the sixth day after onset, was kept in the Military Hospital until the 28th day. As additional patients were added to the series the period of hospitalization steadily diminished from four weeks to about two weeks (see Table III). The average for the entire group was 17.8 days,³ which con-

trasts with an average hospitalization of 29.9 days in the untreated group. There were no complications and no deaths among the treated patients whereas among the untreated persons one died and two others had serious complications.

Patients with other diseases who received chloromycetin

The cases of suspected scrub typhus destined for specific therapy were numbered serially as they came under observation. It is apparent from Table III that the 30 patients with scrub typhus were culled from a series of 49 persons who were originally thought to have tsutsugamushi disease. Treated Patients 44 and 45 who are listed in

³ Treated patients were generally up and about in the hospital and grounds within a few days after becoming afebrile but they were kept in the hospital, although ap-

parently fully recovered, in order to permit medical observation and to prevent their returning to occupational duties.

Table IV suffered from scrub typhus but because of the circumstances surrounding the acquisition of their disease they are not included in the present group. Treated Patients 46 and 47, Gurkah soldiers who acquired scrub typhus during jungle fighting, are reported in detail elsewhere (12) and will not be included in this group. These were the first two persons to receive the synthetic form of chloromycetin. Among the remaining 15 cases who were treated with chloromycetin, two (Nos. 18 and 19) had typhoid fever (13) and three (Nos. 6, 7, and 35) had murine typhus (14), two (Nos. 36 and 42) were infected with *Leptospira icterohemorrhagiae*, and one (No. 37) had malarial hematuria (blackwater fever). No definitive diagnosis was established for the remaining seven patients (Nos. 2, 5, 14, 15, 17, 20, and 38) but all recovered promptly; the contribution of chloromycetin to their recovery could not be assessed. The efficacy of chloromycetin in the treatment of patients with typhoid fever and murine typhus has been commented upon elsewhere (13, 14). Neither of the cases of leptospirosis showed marked benefit from chloromycetin during the 24 to 48 hours it was given, at the end of which time the original tentative diagnosis of scrub typhus was changed, penicillin therapy was instituted, and improvement began shortly. The patient with blackwater fever recovered without clinically apparent kidney damage after institution of antimalarial and alkali therapy.

The treatment of volunteers who contracted scrub typhus in hyperendemic areas

Thirty-seven of the 75 volunteers who were exposed in hyperendemic areas of scrub typhus in the vicinity of Kuala Lumpur during the course of chemoprophylactic tests (15) developed tsutsugamushi disease. In these tests members of the control group developed scrub typhus during the second and third week after initial exposure while those individuals who received the antibiotic prophylactically, during exposure and the succeeding two weeks, remained well until the second week after drug was discontinued. The ultimate attack rate was not significantly different in the prophylactic and control groups of volunteers, and, except for the prolonged incubation period and the absence of eschar formation, there was no

appreciable difference in the disease in volunteers of the two groups.

Data on the 37 volunteers who contracted scrub typhus are given in Table IV where, in addition, information is included on Patients 44 and 45. In general, the volunteers were treated earlier in the course of their disease than were the patients discussed in the previous sections. For example, in a number of instances chloromycetin therapy was instituted on the first febrile day of illness and the average for the 37 volunteers was 3.1 days, which is exactly half that of the treated patients who acquired their disease under strictly natural conditions. Although there was some variation in the amount of drug given and the number of days over which therapy was provided, most of the patients received an initial oral dose of 4.0 gms. followed by a series of seven 0.25 gm. doses given at three hour intervals. The average duration of fever after drug therapy was started was 32.1 hours for this entire group. The averages for each of the four sub-groups consisting of those who had received chloromycetin prophylaxis and the controls in each of two field trials varied between 28.3 and 36.8 hours.

An unusual manifestation was noted in volunteers developing scrub typhus which had not previously been observed in patients who were treated symptomatically or in those mentioned in Table III who received chloromycetin therapeutically. This was the occurrence of relapses in volunteers who had apparently been cured by the initial course of drug. This phenomenon is discussed at some length in a report of the chemoprophylactic trials (15). However, it is of sufficient importance to review at this time. Twenty of the 37 volunteers who contracted tsutsugamushi disease in the two trials developed one or more relapses. The first recrudescence appeared between five to 15 days after the initial onset of disease. Since in most instances the initial therapeutic course had not exceeded 24 hours, the average interval between the last dose of drug used in the initial attack and the onset of the relapse was 7.1 days. Eight of the 20 patients had a second relapse which began on the average 6.5 days after the onset of the first recrudescence and 4.5 days after the end of therapy for the first relapse. Three of the group suffered from a third relapse. As in the first two recrudescences

cences the interval between this and the preceding febrile episode was about one week.

Patients suffering from their first relapse were treated in essentially the same manner, receiving 3.0 to 6.0 gms. of drug during a period of 24 hours or less. The average duration of the recrudescent fever after beginning therapy was 24.3 hours. Those individuals who had second relapses received 3.0 to 5.75 gms. of drug, but the method of administration differed in this group. Patient CP5 received 3.0 gms. given in a single dose. Patient CP1 received 1.0 gm. daily for five days and the others were treated for a period intermediate between the two. Irrespective of the regime these patients became afebrile within a day. The

three patients with third relapses had mild fevers in the range of 100° F. which subsided rapidly whether or not therapy was given.

The diagnosis of scrub typhus was proved by laboratory procedures in all but one of the 37 volunteers who became ill with scrub typhus. These procedures consisted of either isolation of rickettsiae from the blood or the demonstration of the development of a positive Weil-Felix reaction (see Table IV). The initial relapses were proved to be scrub typhus in the majority of cases by demonstration of rickettsemia, *i.e.*, in 11 of the 14 patients on whom attempts at isolation were made. Similarly, one of two patients examined had rickettsemia during his second relapse.

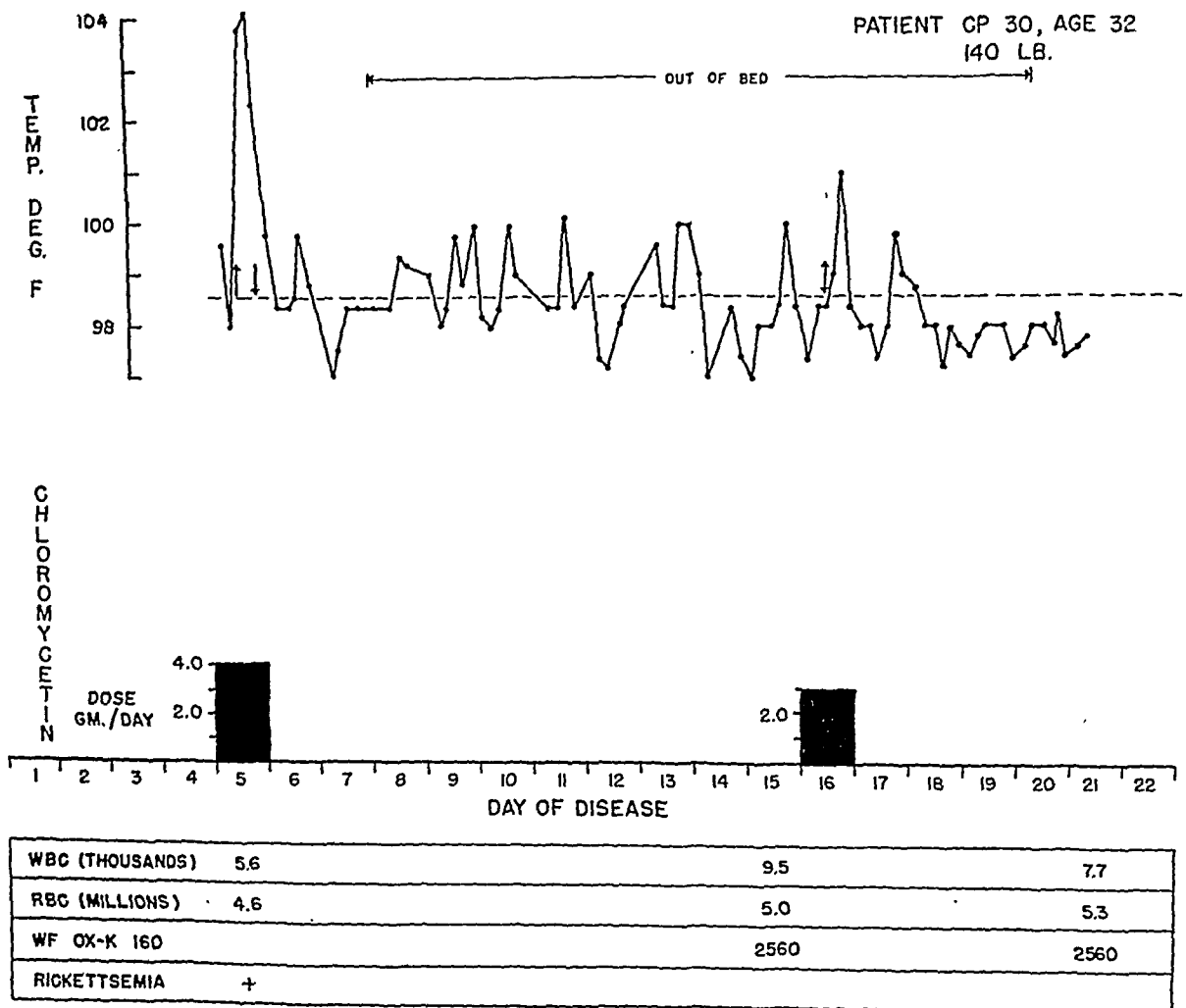


FIG. 5. CLINICAL RESPONSE OF PATIENT CP 30 TO INADEQUATE CHLOROMYCETIN THERAPY BEGUN ON THE FIFTH DAY OF DISEASE

The low grade intermittent fever subsided promptly after the second course of treatment.

TABLE IV

SCRUB TYPHUS IN VOLUNTEERS EXPOSED IN HYPERENDEMIC AREAS, KUALA LUMPUR, 1948

VOLUNTEER				DAYS FROM INITIAL EXPOSURE TO ONSET	DAY OF DISEASE R _x BEGUN	HOURS OF FEVER AFTER R _x	OX-K TITER		ISOL. OF RICK.	RELAPSE			TOTAL DURATION	
NO.	AGE	RACE	TEST GROUP				ACUTE	CONV.		DAYS FROM ONSET ORIG. ILLNESS TO RELAPSE	HOURS OF FEVER AFTER R _x	ADDITIONAL RELAPSES	FEVER (DAYS)	ONSET TO RECOVERY (DAYS)
CP 1	32	E	C	12	2	56	0	40	POS.	12	21	2	11	29
CP 2*	33	E	C	13	1	39	0	160	"	10	20	1	6	17
CP 3	35	M	C	14	2	20	80	640	"	12	16	0	5	13
CP 4	20	M	C	14	2	48	40	40	"	NONE			4	4
CP 5*	31	M	C	14	2	40	0	40	"	12	12	1	14	32
CP 6	20	M	C	15	2	20	40	80	NEG.	10	16	0	6	12
CP 7	33	D	C	15	2	34	40	1290	POS.	5	24	1	8	12
CP 8	30	M	C	15	2	16	0	80	"	NONE			3	3
CP 9*	17	M	C	15	2	44	0	80	"	10	36	0	7	13
CP 10	20	M	C	15	2	20	0	320	"	9	36	0	7	14
CP 11	32	E	C	13	4	28	0	160	"	13	36	0	7	14
CP 12	20	M	C	16	2	40	40	80	"	NONE			4	4
CP 13	22	M	C	16	2	18	40	160	"	15	16	0	5	19
CP 14*	32	E	C	17	3	32	80	1290	"	7	22	2	10	21
CP 15	20	M	C	18	1	32	40	640	NEG.	NONE			2	2
CP 16	19	M	C	18	1	31	40	160	POS.	7	21	0	5	12
CP 21	36	D	C	21	6	36	40	2560	"	NONE			7	7
AV. CP 1-21				15.4	2.2	32.6			88%	GROUP	RELAPSE RATE	71%		
CP 22	22	M	P	31	3	20	40	160	NEG.	NONE			4	4
CP 24	47	A	P	32	1	25	0	160	POS.	8	24	1	6	15
CP 25*	41	A	P	32	1	29	0	320	"	8	14	2	10	23
CP 26*	30	E	P	33	2	40	0	40	"	9	39	0	8	14
CP 27	20	M	P	32	5	18	0	80	"	NONE			6	6
CP 28*	31	E	P	31	5	42	40	640	"	NONE			7	7
CP 29	27	M	P	33	3	34	40	640	"	11	35	0	9	14
CP 30	25	M	P	32	5	48	160	1280	"	8	36	0	15	21
CP 31	20	M	P	32	5	32	80	1280	"	NONE			6	6
CP 32	28	M	P	32	5	16	40	640	"	NONE			6	6
CP 33	20	M	P	34	3	20	0	80	"	NONE			4	4
CP 34	30	M	P	36	2	40	0	40	"	8	16	1	7	14
AV. CP 22-34				32.5	3.3	30.3			92%	GROUP	RELAPSE RATE	50%		
PR 1	19	M	C	10	5	65	40	320	POS.	NONE			8	8
PR 3	15	M	C	14	2	29	0	80	"	NONE			3	3
PR 4	22	M	C	15	5	30	40	2560	"	NONE			6	6
PR 5	23	M	C	15	2	33	40	2560	"	NONE			4	4
PR 6	18	I	C	15	5	27	40	160	"	NONE			6	6
AV. PR 1-6				13.4	3.8	36.8			100%	GROUP	RELAPSE RATE	0%		
PR 8	16	M	P	26	5	46	40	80	POS.	9	68	0	7	16
PR 9	23	M	P	28	3	20	80	640	"	13	21	0	8	16
PR 10	36	J	P	28	8	19	40	160	"	NONE			9	9
AV. PR 8-10				27.3	5.3	28.3			100%	GROUP	RELAPSE RATE	66%		
44	18	M	C+	30 MAY	3	32	40	ND	POS.	NONE			4	4
45	40	M	C+	12 JUNE	4	20	0	320	"	6	8	0	7	10

PATIENTS WITH CP NUMBERS WERE IN TEST NO. 1; THOSE WITH PR NUMBERS, IN TEST NO. 2

RACE: A=AMERICAN, B=BURMESE, E=ENGLISH, I=INDIAN, J=JAVANESE, M=MALAY.

TEST GROUP: C=CONTROL, P=PROPHYLACTIC, ND=NOT DONE

* THESE VOLUNTEERS HAD RECEIVED SCRUB TYPHUS VACCINE

+ THESE EMPLOYEES WERE NEGLIGENT IN THE USE OF REPELLANT AND CONTRACTED SCRUB TYPHUS IN THE HYPERENDEMIC EXPOSURE AREA. THEIR EXPOSURE IS CONSIDERED MORE COMPARABLE TO THAT OF THE VOLUNTEERS THAN TO THAT OF THE OTHER NATIVE WORKERS EMPLOYED IN THIS AREA.

The record of volunteer Patient CP14, which is presented graphically in Figure 4, shows the usual course of the initial disease and the first relapse in the volunteers and the effect of therapy on these febrile episodes. This record also illustrates the findings in most of the individuals who had second and third relapses. It will be seen that this volunteer had a series of relatively mild febrile attacks and that he was ambulatory during most of the time covered by the chart, carrying on desk work but avoiding the more strenuous efforts of field work. The records of Patients CP4 and CP25 resemble that shown in Figure 4 in that these individuals also spent most of their time on duty between febrile episodes.

In contrast to the general rule that the volunteers who suffered from relapses were asymptomatic between their brief recurrent febrile episodes, were the findings in Patient CP30, whose record is summarized in Figure 5. This individual remained well until the 32nd day after the initial exposure which was ten days after his last prophylactic dose of chloromycetin. He was lost from observation from the 30th day after the first field exposure until the fifth day of his illness. At this time he was given 4.0 gms. of drug over a period of eight hours and became afebrile within 40 hours. As will be seen in Figure 5 the resultant

afebrile period was of relatively short duration since low grade intermittent fever occurred during the ensuing week. The temperature reached 101° F. on the 16th day after onset. At this time 3.0 gms. of drug were given in a single dose and the patient became permanently afebrile 40 hours later. It would appear that this was an instance in which the infection was controlled for only a brief period by the therapeutic regime employed. It is probable that the course of treatment used for Patient 1, who received 11.6 gms. of chloromycetin over 120 hours (Figure 2), would have been more appropriate in this instance. It may be mentioned that Patient CP30, along with CP21, spent 24 hours a day for nine days in the infected areas. These two, like the rest of the volunteers, received seven hours of supervised exposure daily but in addition they spent the remainder of the nine days of exposure in the areas serving as guards.

Incidence of eschars in patients with scrub typhus

The incidence of eschars in patients with scrub typhus has varied widely under different circumstances and in different geographic areas. Thus, during World War II the incidence of eschars observed in Allied troops from different combat areas ranged between 40 and 100 per cent (16-18). It

TABLE V
INCIDENCE OF ESCHARS IN PATIENTS WITH
SCRUB TYPHUS, KUALA LUMPUR, 1948

GROUP	RACE	NO. OF CASES	NO. WITH ESCHARS	% WITH ESCHARS
NATURALLY OCCURRING CASES	CAUCASIAN	6	3	50
	ASIATIC	41	3	7
	BOTH	47	6	13
PROPHYLACTIC FIELD TRIAL NO DRUG	CAUCASIAN	4	4	100
	ASIATIC	18	2	11
	BOTH	22	6	27
PROPHYLACTIC FIELD TRIAL DRUG	CAUCASIAN	4	0	0
	ASIATIC	11	0	0
	BOTH	15	0	0
TOTAL	CAUCASIAN	14	7	50
	ASIATIC	70	5	7
	BOTH	84	12	14

may be noted that the absence of this sign delayed the recognition of *R. tsutsugamushi* infections in northern Australia and Malaya (10, 11).

The present observations summarized in Table V reemphasize the marked variation in incidence of eschar formation in different groups of patients. Among the 47 patients with naturally acquired scrub typhus seen in the present study, six were Caucasians and the remainder Asiatics. One-half of the Caucasians developed eschars whereas only 7 per cent of the Asiatics showed this lesion. The frequency with which eschars appeared among the 37 volunteers who acquired scrub typhus following exposure in infected areas likewise appeared to be related to race but, in addition, was dependent upon whether chemoprophylaxis had been received. Thus, none of the four Caucasians and 11 Asiatics who had received chloromycetin prophylactically during the field trial developed eschars with their attack of scrub typhus, whereas all four Caucasians and two of 18 Asiatics who did not receive chloromycetin during their exposure developed an eschar.

These findings agree with earlier observations in Malaya (10, 11) that eschars are more frequently observed in Europeans than in Asiatics. They also indicate that the prophylactic administration of the specific drug during and following exposure influenced the formation of eschars. While no factual data are available to explain the role of prophylaxis in the eschar formation, it has been postulated elsewhere (15) that during the suppression of growth of rickettsiae by the drug, sufficient local immunity developed in the cutis at the site of the infected bite to prevent subsequent development of the eschar.

Unusual cutaneous lesions in volunteers developing scrub typhus

An unusual skin eruption was observed on two of the volunteers (CP2 and CP14) in the first field trial. On the ninth day of exposure these individuals noted a number of small erythematous spots which itched slightly. These occurred in the axillae, groins, popliteal spaces, and around the waist, all areas where mite attachments are most apt to occur. These minor lesions persisted for several days and did not change during the prefebrile period when the primary eschar was developing in these two volunteers. Shortly after

the onset of fever, when the primary eschar was already well developed, vesicles having a diameter of 2 to 4 mm. and a height of 1 to 2 mm. suddenly blossomed on the erythematous lesions. These looked like early varicella pocks but fluid could not be obtained when aspiration was attempted. Each volunteer had 50 to 100 vesicles which were limited almost entirely to the trunk, occurring primarily in the axillae and groins, and around the waist. The pock-like lesions receded a day or so after chloromycetin therapy was instituted but again flared up during the first relapse of both volunteers only to subside again when the fever disappeared. One of the volunteers, who was a physician, was convinced that at least six of the vesicles developed on marked sites where mites had been observed attached.

Neither histological studies nor attempts to recover rickettsiae from the lesions were undertaken. It is not possible to say whether these lesions represented unusual allergic reactions to mite bites which became infected during the rickettsemic phase of the disease; or whether they represented aborted primary eschars, occurring at the site of bites of infected mites acquired late in the exposure period, which were controlled by chemotherapy; or whether they were unrelated to scrub typhus.

Chloromycetin in blood, spinal fluid, and milk

The blood level of chloromycetin was determined on a number of occasions in each of the treated patients listed in Table III. Similar determinations were also made on selected patients with scrub typhus from the group of volunteers. There appears to be little point in discussing the blood levels in detail. In general, the data presented in Figures 2 and 3 illustrate the types of curves obtained in the prolonged and short courses of therapy.

The levels of chloromycetin in blood and spinal fluid specimens taken simultaneously were determined in three instances. In addition, on two occasions specimens of blood and milk, obtained at the same time from one of the lactating patients, were assayed. The results summarized in Table VI indicate that the level of chloromycetin in the spinal fluid is about half that found in the blood and that similarly the levels in the milk are of this same order of magnitude.

TABLE VI
COMPARATIVE CHLOROMYCETIN LEVELS
IN BLOOD, SPINAL FLUID AND MILK

PATIENT NO.	DISEASE	BLOOD LEVEL μG./CC.	C.S.F. LEVEL μG./CC.	MILK LEVEL μG./CC.
18	TYPHOID	46	20	
19	TYPHOID	21	9	
37	MALARIA	37	10	
24 (8 May)	SCRUB	49		25
24 (9 May)	"	26		16

Rickettsemia during afebrile periods

The present work brought out a fact not previously recognized, namely, that *R. tsutsugamushi* may occur in the blood at times when a patient is afebrile and essentially asymptomatic. Patient CP14, one of the volunteers in the prophylactic trial, developed a lesion in the pretibial region of his left leg on the 15th day after initial exposure

in the field. This vesicular lesion with a surrounding areola increased in size during the next few days while the patient remained ambulatory. Enlargement of the left inguinal nodes developed, and on the fifth day after its appearance the pretibial lesion was a typical eschar with a black center. It will be seen from Figure 4 that the patient had an elevation of temperature for the first time on the afternoon of the third day after the eschar was noted. A sample of blood obtained on the morning of this day contained *R. tsutsugamushi*. The rickettsial agent was again isolated from blood taken immediately before chloromycetin therapy was begun on the third febrile day of the illness. This is the first instance on record in which rickettsiae have been demonstrated in the blood prior to the febrile phase of scrub typhus.

From the very beginning of the present work we were interested in determining how rapidly rickettsiae disappeared from the blood of treated patients. Therefore, in treated Patient 1 blood samples were obtained at frequent intervals during the first two and one-half days after therapy

TABLE VII
PERSISTENCE OF RICKETTSEMIA AFTER TREATMENT
WITH CHLOROMYCETIN

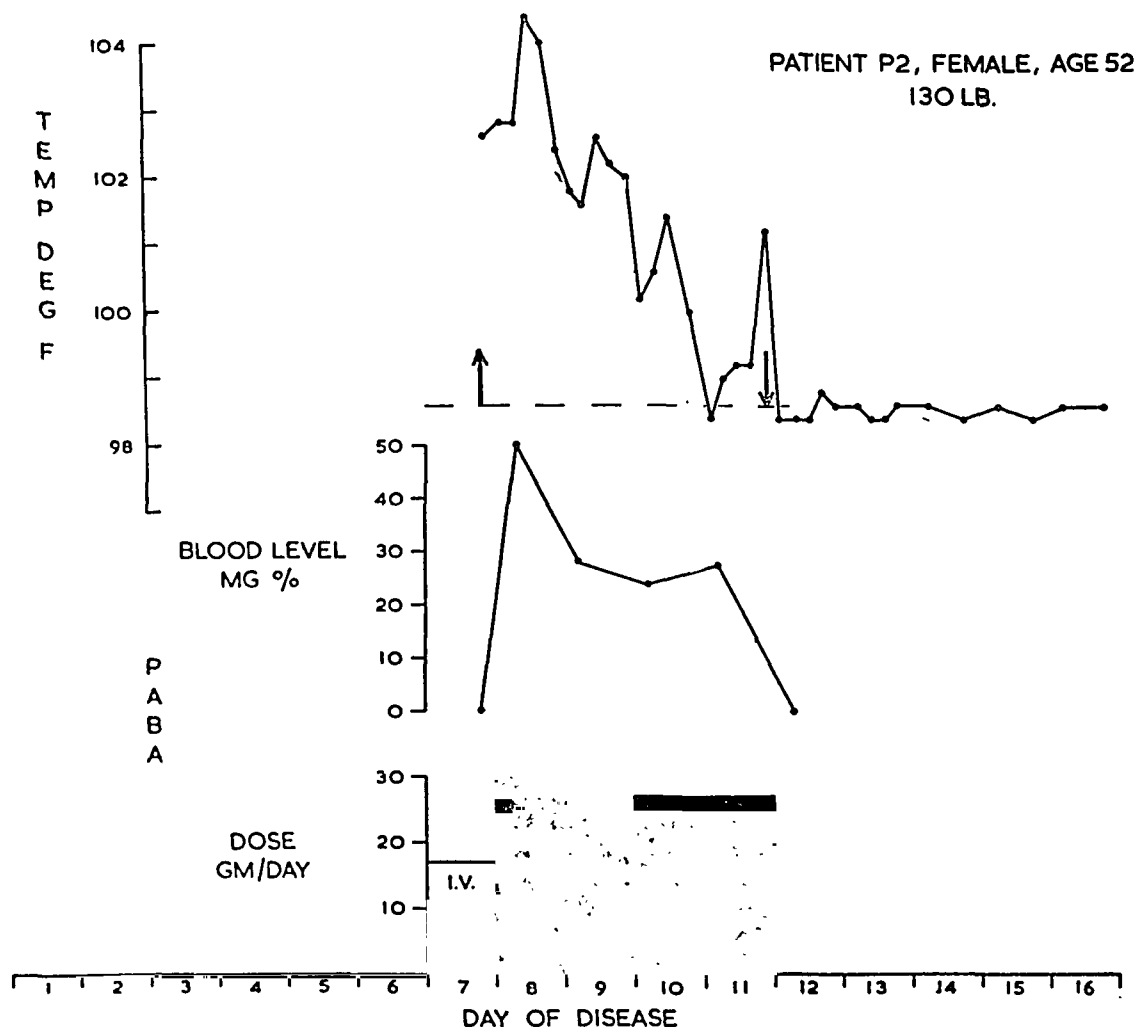
SCRUB TYPHUS CASE NO.	SPECIMEN TAKEN		CHLOROMYCETIN BLOOD LEVEL μG. / CC.	RICKETTSSIAE IN BLOOD
	HOURS AFTER Rx BEGUN	PT'S. TEMP. AT TIME		
1	PRERx	102.8	0	POS.
	4	103.0	27	POS.
	8	100.8	28	POS.
	12	101.4	16	POS.
	23	98.4	21	POS.
	30	98.0	13	POS.
	54	97.8	8	NEG.
4	PRERx	102.0	0	POS.
	6	101.4	36	POS.
29	PRERx	102.2	0	POS.
	17	99.0	45	POS.
	42	98.0	7	NEG.
43	PRERx	101.8	0	POS.
	28	98.6	19	POS.
49	PRERx	100.2	0	POS.
	1/2	101.6	15	POS.*
	2	100.4	28	POS.
	6	102.6	100	POS.
	10	101.4	88	POS.
	24	98.6	26	POS.
	30	97.4	8	POS.
	48	97.4	0	NEG.

* THE MLD₅₀ TITER OF THIS SPECIMEN IN MICE WAS 10^{-1.5}

was begun and were used for isolation studies and for determination of chloromycetin blood levels. The data on this individual and on four other patients with scrub typhus who were investigated along these lines are given in Table VII. Several points are evident from the tabular material. In the first place, rickettsemia was demonstrated in three patients 28 to 30 hours after therapy was

begun at which time the patients were afebrile. However, the rickettsiae had disappeared from the blood of these three patients 42 to 54 hours after therapy was instituted.

It has been shown that chloromycetin, even in concentrations of 1250 $\mu\text{g.}$ per cc., has no *in vitro* rickettsiocidal activity against the agent of tsutsugamushi disease (19). Therefore, the presence



RASH	+	±	0							
ESCHAR	++	++	++	++	+	+	+	+	+	±
WBC (THOUSANDS)	8.6			9.7					4.0	
HGB (GM)	12.2			12.0					10.7	
WF OX-K	40								1280	
RICKETTSEMIA	+		+							

FIG. 6. CLINICAL RESPONSE OF PATIENT P2 TO PABA THERAPY BEGUN ON THE SEVENTH DAY OF DISEASE

A total of 127.5 gms. of sodium p-aminobenzoate was given over a period 100 hours.

of concentrations of 50 to 100 μ g. per cc. of drug such as occurred in the patient's blood samples would not be expected to inactivate the organism. Furthermore, the relatively small amounts of drug present in the material inoculated into mice could not be expected to influence the infection in these animals.

Para-aminobenzoic acid in the treatment of scrub typhus

Para-aminobenzoic acid has been used with definitely beneficial results in the treatment of a number of rickettsial diseases (20, 21) including scrub typhus (22). Clinical results with this drug are less striking than are those obtained with the new antibiotic; nevertheless, when used relatively early in the disease, the duration of the febrile period and the mortality are reduced. Several patients with scrub typhus were treated with p-aminobenzoic acid while under our observation during the present work. The record of one of these, Patient P2, is illustrated in Figure 6. The results obtained in this individual correspond in general with those observed in the other two. The patient was admitted on the evening of the seventh day and at 8:00 p.m. received 6.0 gms. of sodium p-aminobenzoate intravenously. Between then and midnight she was given an additional 11.0 gms. of the drug by mouth. During the succeeding four days she received 25–30 gms. of sodium p-aminobenzoate daily. An adequate blood level was reached and maintained. The fever disappeared by lysis over four days and after the 12th day the patient remained afebrile. In general, the results in our hands agreed with those obtained by Tierney (22) in the treatment of 18 patients with scrub typhus in Assam.

It is our opinion that p-aminobenzoic acid has a place in the treatment of this disease when the newer therapeutic agents are not available.

Lack of toxic manifestations attributable to chloromycetin in treated patients

No significant manifestations of toxicity were attributable to the use of chloromycetin in the treatment of 84 patients mentioned in this report; this includes not only 69 patients with scrub typhus but the additional group of 15 with other diseases. The occurrence of mild cerebral stimulation in persons receiving single 4.0 gm. doses of drug has

been mentioned elsewhere as has the fact that the administration of 12.0 to 20.0 gms. of drug over a period of three weeks to 34 volunteers in the chemoprophylactic studies was without untoward effect (15). Most of the treated patients who had acquired their scrub typhus under strictly natural conditions received considerably smaller amounts of drug than did those volunteers in the chemoprophylactic tests who suffered from relapses. Even in the latter group no evidence of toxicity was demonstrated. It may be mentioned that certain of these volunteers, during the course of their prophylaxis and throughout their treatment, received 38.0 to 46.3 gms. of drug over a period of about eight weeks (see Table VIII). The administration of such large amounts of drug over an appreciable period of time indicates that patients are not readily sensitized to this substance. Even those individuals who received the compound intermittently remained free of evidence of sensitivity.

Red blood cell counts (or hemoglobin levels) and leukocyte counts were done, in so far as possible, both before treatment and during convalescence on all patients suffering from scrub typhus who received chloromycetin therapeutically. In five instances, when severely ill patients were treated in estate hospitals, it was not practicable to obtain pretreatment values. In the evaluation of data obtained in this fashion, it may be pointed out that a significant drop in red blood cell count, *i.e.*, 0.9 million cells or 2.5 gms. of hemoglobin, occurs during the first two weeks of the untreated disease (23).

Of the 64 patients who received chloromycetin therapy for scrub typhus and who were adequately studied, 27 showed a drop in red blood cell count. The maximum decrease observed for any one patient was 1.10 million, and the average decrease for the entire group of 64 was 0.11 million. These reductions in red blood cell count are of the same order as those occurring in untreated patients (23). In the present group, there was an average rise of leukocyte count from 6,700 to 7,400; this also occurred frequently in untreated cases during convalescence. It is interesting to note that chloromycetin was administered to one patient whose leukocyte count at the beginning of therapy was only 1,700. His recovery was uneventful, and, at the time of discharge, the white

TABLE VIII
RELAPSES IN SCRUB TYPHUS PATIENTS: INTERVALS AND TREATMENT, KUALA LUMPUR, 1948

VOLUNTEER NO.	ORIG. ILLNESS		1st. RELAPSE			2nd. RELAPSE			3rd. RELAPSE			TOTAL AMOUNT DRUG REC. INCLUDING PROPHY - LAXIS (GM)
	TREATMENT		DAYS FROM ONSET ORIG. ILLNESS TO ONSET OF 1st. RELAPSE	TREATMENT		DAYS FROM ONSET 1st. RELAPSE TO ONSET OF 2nd. RELAPSE	TREATMENT		DAYS FROM ONSET 2nd. RELAPSE TO ONSET OF 3rd. RELAPSE	TREATMENT		
	TOTAL (GM)	DURATION (HR)		TOTAL (GM)	DURATION (HR)		TOTAL (GM)	DURATION (HR)				
CP 1	10.75	48	12	4.0	8	10	5.0	96	6	NONE		19.75
CP 2	9.0	40	10	8.0	36	6	3.0	72	NONE			20.00
CP 3	5.75	28	12	4.0	8	NONE						9.75
CP 5	5.75	22	12	3.5	7	11	3.0	SINGLE DOSE	NONE			12.25
CP 6	5.75	22	10	4.0	8	NONE			NONE			9.75
CP 7	5.85	22	5	3.0	SINGLE DOSE	5	4.25	10	NONE			13.10
CP 9	3.5	SINGLE DOSE	10	4.0	8	NONE						7.50
CP 10	4.0	SINGLE DOSE	9	4.75	1	NONE						8.75
CP 11	4.5	12	13	4.0	4	NONE						8.50
CP 13	4.0	SINGLE DOSE	15	4.0	8	NONE						8.00
CP 14	5.25	14	7	4.0	8	4	4.0	72	7	5.0	96	18.25
CP 16	4.0	SINGLE DOSE	7	4.0	8	NONE						8.00
CP 24	8.0	84	8	5.0	8	6	3.0	8	NONE			38.00
CP 25	9.5	90	8	4.0	48	5	5.75	100	5	5.0	120	46.25
CP 26	11.5	78	9	5.75	24	NONE						39.25
CP 29	4.0	6	11	5.75	21	NONE						31.75
CP 30	4.0	8	8	3.0	SINGLE DOSE	NONE						29.00
CP 34	4.0	12	8	4.0	6	5	NONE					30.00
PR 8	5.75	21	9	4.0	SINGLE DOSE	NONE						21.75
PR 9	5.75	21	13	5.75	21	NONE						23.50
AV.	6.0		9.8	4.4		6.5	4.0		6.0	5.0		

count had risen to 4,200. Routine urine analyses, performed during the acute illness and convalescence, demonstrated no abnormalities that were not present before therapy was initiated. In summary, no significant change of the blood or urine attributable to the administration of chloromycetin was noted.

DISCUSSION

The observations reported at this time clearly indicate that chloromycetin is a highly effective therapeutic agent in the treatment of scrub typhus in man. When properly used, it may be expected to produce obvious improvement in the patient's condition in a matter of a few hours and to render him afebrile in about 30 hours.

The short course of therapy which was used extensively in the present studies consisted of an initial oral administration of approximately 60 mg./kg. of body weight which was followed by an additional 1.5 to 2.0 gms. of drug given as 0.25 gm. tablets at three-hour intervals. This regime appeared to be satisfactory in those patients who acquired their disease during exposure while pursuing their normal occupational duties. On the average these patients were first treated 6.2 days after onset of illness. These individuals all became afebrile in an average of 31.8 hours and remained afebrile thereafter.

The results in volunteers who were purposely exposed in hyperendemic areas of scrub typhus during the course of chemoprophylactic field trials differed from that observed in the patients who acquired the disease during normal occupational exposure in infected areas. Fifty-four per cent of the volunteers who developed scrub typhus subsequently had one or more relapses following the apparent cure which had resulted from the initial treatment. Although the volunteers who had relapses became permanently afebrile, on the average, on the 17th day after onset of original illness, nevertheless, a number of them did not return to full strength and vigor until two or three weeks later. Various factors which might have accounted for the relapse rate in the volunteers have been discussed at length in another paper (15). Among these factors only two that need be mentioned here are (a) the infecting dose was undoubtedly heavy and was probably repeated over a period of days in the volunteers, and (b) treat-

ment was begun appreciably earlier in these persons than in those who acquired their infection naturally. The hypothesis which is presently preferred to account for these relapses is as follows: "The rickettsiostatic effect of chloromycetin was dissipated within a short time after therapy was stopped. At this early stage of infection the immune mechanism of the host had not yet had time to respond adequately to the rickettsial agent. Therefore, after a temporary interruption due to drug, the growth of the rickettsiae proceeded at an unabated rate. Finally, after an interval of five to 10 days, the rickettsial multiplication reached a level sufficient to elicit clinical manifestations again" (15).

The variation in incidence of eschars in patients of different races has been previously observed with scrub typhus (10, 11b) and was the experience in the present studies on persons who acquired the disease during normal occupational duties. Furthermore, the high incidence of eschars in the Caucasians in the control chemoprophylactic group and the relatively low incidence in the Asiatics in this group was to be expected. However, the absence of eschars was an unexpected observation in those persons who developed scrub typhus after chemoprophylaxis was discontinued. It has been suggested that local cutaneous immunity developed in these persons during the subclinical infection which was brewing during the course of prophylaxis.

SUMMARY AND CONCLUSIONS

Sixty-nine patients with scrub typhus were treated with chloromycetin. Although some of these were desperately ill when therapy was instituted, there were no deaths in this group. The mortality rate among the 19 untreated patients observed during the present work was slightly greater than 5 per cent; this agrees well with the figure of 6.7 per cent in a series of 164 patients observed by Lewthwaite in Malaya (11a).

The most commonly used therapeutic regime in the present work employed about 6.0 gms. of chloromycetin orally over a period of 24 hours. This was adequate to render patients, who acquired their disease naturally, afebrile within an average of 32 hours irrespective of the time during the course of the disease when the drug was started. These patients convalesced rapidly and had no

relapses. In contrast 54 per cent of the 37 volunteers who acquired scrub typhus developed relapses; these persons had been exposed for a number of days in hyperendemic areas during chemoprophylactic field trials. It is apparent that the course of therapy given early in the disease to volunteers was not adequate to control permanently the infection in them. However, the recrudescence of disease was controlled without difficulty when chloromycetin was again administered. Additional information is needed before final conclusions can be drawn about the optimal therapeutic regime. In the meantime, it is recommended that all patients with scrub typhus, irrespective of the time when treatment is started, be given an initial oral dose of 60 mg./kg. of body weight and that this to be followed by 0.25 gm. doses of drug at three-hour intervals for at least 24 hours. If a recrudescence of fever occurs, the course should be repeated.

Chloromycetin is of low toxicity for man. No significant untoward effects were observed in any of the 84 patients who received the drug in the current studies.

Chloromycetin appears in the spinal fluid and milk of human beings reaching levels about half that in the blood.

Rickettsemia may occur for short periods before fever begins in tsutsugamushi disease and for a number of hours after treated patients become afebrile.

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CHANGES IN LIVER GLYCOGEN STUDIED BY THE NEEDLE ASPIRATION TECHNIC IN PATIENTS WITH DIABETIC KETOSIS. WITH A METHOD FOR THE ESTIMATION OF GLYCOGEN FROM HISTOLOGIC PREPARATIONS¹

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Previous attempts to estimate the glycogen content of the human liver were based on determinations performed on material obtained by operative biopsy or from postmortem specimens. Observations on animals have shown that these procedures may introduce an important error through the effect of the commonly used anesthetic agents, or of trauma, before the specimen is obtained. The development of a technic for the removal of small specimens of liver by aspiration biopsy is well adapted to the study of glycogen metabolism, for it avoids the uncertain effects of premedication, general anesthesia and the trauma of operation. By this method serial biopsies of the liver have been obtained in a study of patients under treatment for diabetic acidosis.

METHODS

Liver tissue was obtained by needle biopsy, using the Vim-Silverman needle, under local procaine infiltration. With practice, it was possible to obtain an adequate specimen in less than three seconds from the time of insertion of the needle into the substance of the liver. Speed was desirable, since patients in severe acidosis were unable to hold their breath. The specimens were immediately fixed in iced absolute alcohol or Rossman's fixative.

Plasma carbon dioxide combining powers were determined by the method of Van Slyke and Neill (1). The Folin-Wu method was used for the determination of blood glucose (2).

THE TECHNIC FOR THE ESTIMATION OF HEPATIC GLYCOGEN

At first, the glycogen concentration was determined by the chemical method of Good, Kramer and Somogyi (3). This technic was unsatisfactory, however, since without histologic examination it was impossible to be

certain that the entire specimen submitted to analysis consisted of hepatic parenchyma. On several occasions, specimens obtained by needle biopsy have included bits of skeletal muscle, fat or fibrous tissue with the liver specimen. Had the entire contents of the needle been analyzed, the results would not have been representative of liver glycogen. It therefore seemed essential to examine microscopically the material which was to be analyzed. To do this, some histochemical method of estimating glycogen had to be adopted.

It had previously been shown by Deane, Nesbitt and Hastings (4) that the staining intensity of glycogen in rat livers was proportional to the concentration of glycogen determined by the usual chemical methods. This fact was used to develop a method of estimating the glycogen content of the specimens obtained by biopsy.

The tissue was prepared and stained for glycogen by the method of Gomori (5) under carefully controlled uniform conditions. No counterstain was used. Control slides prepared from tissue digested by salivary amylase demonstrated that only glycogen-containing material was stained by this method.

The optical density of the stained glycogen was determined photometrically. A photomicrographic apparatus was set up in a dark room. The slide to be examined was placed upon the mechanical stage and its image projected, at a magnification of approximately $500\times$ upon a ground glass screen with a central clear zone. Over the clear window was fixed the aperture of the photoelectric cell of a Photovolt electronic photometer. With the substage lamp turned off, the photometer was adjusted to read "0.0." The lamp was then turned on, and the slide scanned in an area without tissue. The substage diaphragm was then adjusted so that the photometer reading was "100.0." This step automatically corrects for variations of color or thickness of the slide, coverslip or mounting medium. The image of the preparation was then moved across the screen so that successive random readings of the light transmitted through the tissue could be made. Because of the manner of adjustment, the galvanometer readings represented "per cent transmission." These readings were translated to optical density by the application of the formula: $d = 2 - \log T$. Unstained tissue had no detectable optical density. Since glycogen stains black by the method used, it was possible to make readings using a white source of light without filters. A method similar to this has

¹ Presented in part at the Southern Section, American Federation for Clinical Research, New Orleans, La., January 29, 1948, and at the American Physiological Society, Atlantic City, N. J., March 18, 1948.

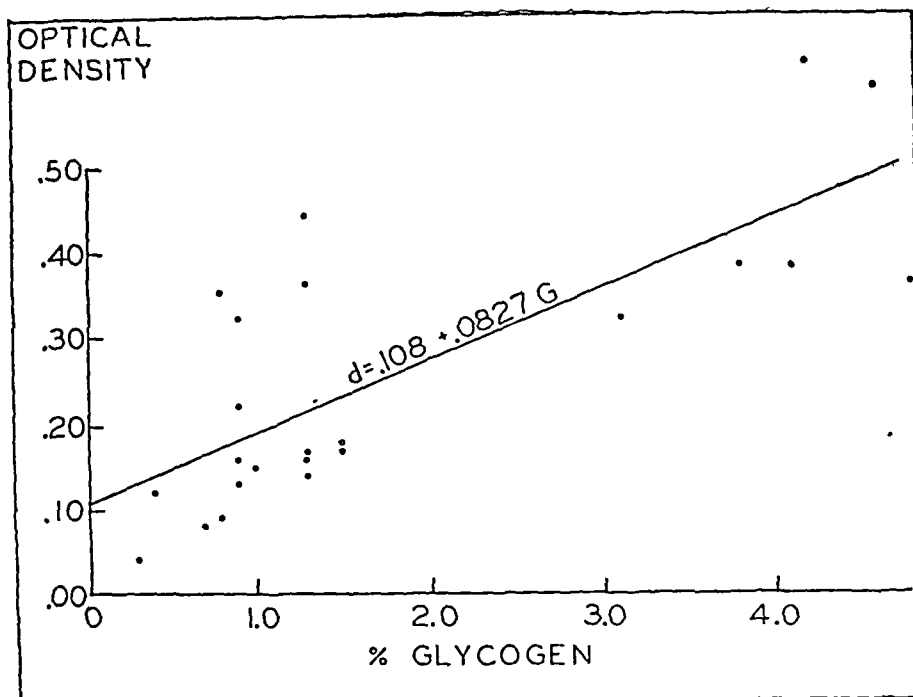


FIG. 1. RELATIONSHIP OF OPTICAL DENSITY OF HISTOCHEMICAL PREPARATION TO GLYCOGEN CONTENT OF RAT LIVERS

been described by Buchanan and Hill for the estimation of myelin (6).

It has been observed by Gomori and Goldner (7) that there may be considerable focal variation of the glycogen content of liver cells. Hédon and Loubatières, however, analyzing fairly large aliquots of liver, have shown (8) the maximal variation within the liver to be less than 10 per cent. It was necessary, therefore, to determine the mean of a series of readings for each specimen in order to obtain an adequate sampling of the glycogen content of the specimen. The minimum number of observations which would afford the desired accuracy was ascertained by making 100 random readings of a single specimen. With 100 readings, the mean was 0.39 with a standard error of ± 0.012 optical density units. With 20 readings, the standard error ranged from .014 to .022. Ten readings produced standard errors ranging from 0.026 to 0.043. It was felt that the standard error of less than 10 per cent provided by 20 readings was sufficiently accurate for the purposes of the present investigation.

In order to estimate the accuracy of the method, a series of determinations were made comparing the chemical methods of assaying glycogen with the results of the histochemical estimation on the same specimen. A group of rats was injected with glucose whereas others were fasted for various periods to provide liver tissue with varying glycogen contents. The animals were then sacrificed. Immediately after death, adjacent specimens of liver were obtained for the determination of glycogen by the method of Good, Kramer and Somogyi (3) and for histochemical preparations. The regression of optical

density on the chemical glycogen value was calculated. The significance of the regression was determined by dividing the slope by its standard error, giving a value of p^2 of less than 0.01 (Figure 1). The standard deviation of the points about the regression line was ± 0.149 . It is of some importance that the standard deviation of the 100 readings performed on a single preparation was ± 0.121 . In two separate sets of data, the degree of variance caused by the method of observation appears to be almost the same, indicating that the technic is uniform and the results reproducible.

A further conclusion may be drawn from the close agreement of the standard deviations of the regression curve and of a single preparation examined many times. Since the variance of the points about the regression curve is no greater than the variance of a series of optical densities determined for a single specimen, it appears that the entire scatter of points on the regression curve can be accounted for by the limitations of the method of determining optical density. It is therefore unnecessary to suppose that part of the scatter may be caused by unequal distribution of glycogen through the liver. Apparently the mean of 20 readings of the histologic section offers a sample adequate for controlling local variations of glycogen. The limiting factor of accuracy therefore appears to arise, not from the unequal distribution of liver glycogen, but from the limitations of the scanning technic.

²" p " estimated from Fisher's table of p (23). A significant value is considered to be one less than 0.02. Slope = $.0827 \pm .0031$.

TABLE I

Therapeutic procedures and chemical determinations in six patients with diabetic ketosis

Patient	Age Sex	Pre-B ₂ plasma CO ₂ comb. power	Therapy until second biopsy				Liver glycogen gm./100 gm. liver			
			Insulin	Glucose	Saline	Interval to second biopsy	Before B ₂	After B ₂	Increase	Increase
G. H.	28 M	vol. % 14.3	units*	gm.	liters	hours	0.4			gm./100 gm./hr.
			No second biopsy was obtained							
P. C.	26 F	19.1	100 50	100	2.0	1.00	0.3	0.6	0.3	0.3
M. S.	70 F	48.7	45†	Lunch	0.9	2.00	3.5	4.3	0.8	0.4
Gr. H.	35 F	13.9	150 50	150	4.0	2.75	7.0	8.0	1.0	0.4
Br. T.	20 M	38.5	150	100	3.0	3.75	1.9	3.7	1.8	0.5
B. T.	40 F	27.8	250 125	300	8.0	6.00	0.5	4.8	4.3	0.7

* The upper figure refers to total dosage; the lower figure is the amount given intravenously in the clyses. Unless otherwise specified all insulin was regular insulin.

† Administered as a routine injection of protamine zinc insulin five hours before the biopsy.

The standard error of the glycogen determination is approximately ± 0.022 . The limitation of the ability of the method to distinguish differences of glycogen concentration is, therefore, $\pm 0.044 d$ in the regression equation, $d = 0.108 + 0.0827 G$, or a difference of glycogen concentration of 0.5 gm./100 gm. of liver. The sensitivity of this technic is therefore limited, since it cannot detect small alterations of concentration. This disadvantage seems to be outweighed by the opportunity to examine under the microscope the tissue which is to be analyzed, and by the assurance that it is the glycogen concentration of the liver cells which is actually being determined.

New sets of histologic preparations were made from the same blocks of rat liver for repeated histochemical assays. It was found that the slope of the regression curve varied somewhat in the different sets. In spite of this, the relationship of the various samples to one another remained constant. The variation of the slope therefore appeared not to be due to errors of the method of determining optical density, but to variations in the intensity of staining reaction in successive runs of slides. To control this, it was necessary to set up a new reference curve for the calibration of each batch of histologic preparations. This was done by running sections from four of the rat liver preparations previously described along with the biopsy specimens, taking care to treat both standards and unknowns in a uniform manner with regard to sectioning, staining and mounting. The optical densities of the standards were plotted, an estimated regression line drawn, and the glycogen content of the unknown specimens read from this line. All of the biopsies from a given subject were analyzed in the same batch.

CLINICAL MATERIAL

Six patients admitted to the medical service of Grady Memorial Hospital with diabetic acidosis of varying degrees of severity were subjected to needle biopsy of the liver before the institution of treatment. In five cases a second biopsy was obtained after varying periods of therapy. No ill effects attributable to biopsy were observed. The treatment used consisted of the administration of insulin, glucose and salt solutions. The details are given in Table I.

Liver biopsies by the same technic were also done on three patients hospitalized for various acute illnesses not involving the liver or carbohydrate metabolism. The first biopsy was done under basal conditions and was followed by another biopsy in two of these patients after they had been given a standard breakfast which supplied more than 50 gm. of carbohydrate. At the time of these examinations, all patients were convalescent and had been afebrile for more than 48 hours. All had been eating a diet containing more than 300 gm. of carbohydrate for three or more days before the test.

RESULTS

The values obtained in the normal basal individuals were 2.8, 3.2 and 4.7 gm./100 gm. These values are somewhat higher than the mean value of 2.0 per cent found by Young, Abels and Homburger (9) who obtained their specimens during laparotomy, and by Hildes, Sherlock and Walshe

(10) who employed the aspiration biopsy technic and used a chemical technic for analysis of glycogen content. The present figures, however, fall within the range of normal observations obtained in both of the other normal series. Two subjects were examined one hour after breakfast. In these instances there was an increase of glycogen content of 1.5 and 2.3 gm./100 gm. No comparable observations are available in intact human beings, since the results of Young, Abels and Homburger after the administration of glucose were obtained from patients other than those furnishing specimens prior to carbohydrate administration.

The glycogen content of the livers of patients with untreated diabetic ketosis ranged from 0.3 to 7.0 gm./100 gm. (Table I). If patient Gr. H. is omitted from the calculation, there is a correlation between the carbon dioxide combining power and the liver glycogen concentration. The justification for the elimination of patient Gr. H. from this series is discussed below.

In each patient there was an elevation of the liver glycogen after treatment. If the rate of increase of glycogen in gm./100 gm./hr. is calculated for each patient, and compared with the duration of treatment before the second biopsy, a steady increase in the rate of glycogen deposition is found from a minimum of 0.3 gm./100 gm./hr. after one hour of treatment, to a maximum of 0.72 gm./100 gm./hr. after six hours of therapy.

DISCUSSION

The finding of normal values for the glycogen content of the liver under basal conditions presents additional evidence of the validity of the method used for estimating glycogen in the present study. The fact that the values range somewhat higher than the mean values of other series may be fortuitous in view of the small number of observations. It should be noted, however, that in the series presented by Young *et al.* (9) the patients received "routine preoperative preparation," which may have included morphine. Most of their specimens were obtained during laparotomy under ether anesthesia. The absence of pharmacologic and psychic stimulation prior to biopsy in the present series may account for the somewhat higher glycogen values in our patients.

The livers of untreated diabetic patients contained less glycogen than normal, except in two

cases. One patient, M. S., was mildly decompensated. Her carbon dioxide combining power was almost normal (48.7 vol. per cent), and she recovered spontaneously under the influence of the protamine zinc insulin which she had taken five hours previously. The other patient, Gr. H., may represent a special case, and is discussed below. If this patient is omitted from consideration, there is a correlation between the carbon dioxide combining power and the liver glycogen concentration ($r = 0.939$; p less than 0.02). It therefore appears that the degree of glycogen depletion may have been related to the severity of the acidosis in five of the six patients.

It has generally been stated that the liver glycogen tends to be depressed in diabetic acidosis (11). It has been shown, however, that the liver glycogen of the pancreatectomized dog (12) or alloxanized rat (13, 14) or rabbit (14) may be normal at times when the animal shows signs of severe diabetes. Glycosuria, hyperglycemia and ketonuria may be found in the presence of normal or elevated liver glycogen concentrations. In fact, alloxan-diabetic rats tend to maintain their liver glycogen reserves better during fasting than the normal rat (15). Only when the animal enters a stage of terminal acidosis does the glycogen level fall (16).

Previous studies of liver glycogen concentration in human diabetics have been confusing because the tissue was examined only after death (17, 18) or because, in many cases, the specimens were obtained after treatment with insulin (19, 20). Hildes *et al.* (10), using a technic similar to that used in the present study, determined the glycogen content of the liver of several diabetic patients. Their subjects, although ketonuric, were not severely decompensated, since there was only one in the group with a carbon dioxide combining power of less than 49 vol. per cent. In their series, the liver glycogen concentrations were normal.

The present series represents the first group of human diabetics in severe acidosis whose liver glycogens have been estimated prior to treatment. The relationship between the severity of acidosis and the depression of liver glycogen appears to agree with the results obtained in animals. It is of interest, however, that one patient (Gr. H.) had an elevated liver glycogen concentration while in severe acidosis. Thus, in humans as in ani-

mals, it is possible to have advanced diabetic ketosis in the presence of an elevated liver glycogen concentration.

There is probably, therefore, no causal relationship between depression of liver glycogen and the development of acidosis in diabetes. On the contrary, the nausea and vomiting of ketosis may so restrict the food intake as to play a significant role in the development of liver glycogen depletion. We have no evidence upon which to decide which factor is primary.

After the institution of treatment, there was an increase in the hepatic glycogen concentration in all patients examined. This effect of therapy had previously been noted, in a qualitative observation (21). Following the institution of therapy, the rate of glycogen accumulation by the liver increased over a seven-hour observation period. This finding agrees with the studies of splanchnic glucose balance in human beings during treatment for diabetic ketosis (22). In these patients, there was a latent period of approximately one hour after the administration of large amounts of insulin intravenously before the splanchnic system began to retain glucose. It appears probable that even after the administration of large doses of insulin and glucose, some time must elapse before the liver achieves its maximal rate of glycogen deposition.

One patient, Gr. H., had a liver glycogen concentration far higher than any of the others, and higher than any of the normal subjects in this or other series (9, 10). There can be no doubt that the patient was suffering from severe diabetic acidosis (see appended case history). In certain respects, however, she was different from the other patients in the series. She was very obese, while the other patients were thin or normal in nutrition. The history of polyuria and polydipsia extended back for a period of two years; but she had felt well until, following a trauma, she suddenly developed ketosis. Her acidosis was moderately insulin-resistant, requiring more than 800 units in 24 hours; but when placed on a stringent reducing diet, she lost 20 lbs. and was controlled well without insulin. The other patients in this series had taken insulin for prolonged periods, and could not be controlled by diet alone. It therefore seems that patient Gr. H. falls into the group of mild diabetics with obesity. The other patients appear to have suffered from a more severe and,

possibly, a basically different type of disease. It may be that this difference explains the fact that, of all the patients examined, only patient Gr. H. had an elevated liver glycogen concentration during severe ketosis.

SUMMARY

The liver glycogen content of diabetic and non-diabetic patients has been estimated by a method of determining the optical density of histologic preparations. The tissue was obtained by needle aspiration and was prepared by Gomori's histochemical technic. The method was sensitive to changes in glycogen concentration of 0.5 gm./100 gm. and appeared to furnish an adequate estimate of the glycogen of the entire liver.

Glycogen determinations were made on the livers of three non-diabetic fasting patients, and in two of them after breakfast. The fasting values ranged from 2.8 to 4.7 per cent. A significant increase in the liver glycogen was observed after a meal.

Six decompensated diabetic patients were subjected to biopsy. In all cases except one, there was a decrease of liver glycogen proportional to the severity of the ketosis. After treatment, all patients showed an increase of liver glycogen.

One patient had a liver glycogen concentration of 7.0 per cent during severe ketosis. Because of her obesity and low insulin requirement, it is suggested that she may have had a type of diabetes different from the other subjects. In spite of her high initial glycogen level, this patient also deposited glycogen in her liver during therapy.

APPENDIX

Gr. H., 27 year old negress, was admitted to the medical service of Grady Memorial Hospital on February 14, 1948, because of abdominal pain. She had noticed frequency of urination, beginning in 1945, and had moderate increase in thirst from that time. One week before admission she had been kicked in the abdomen. Following this, she developed abdominal pain, increased thirst and urination. Three days prior to admission she began to vomit. Eight hours before admission she developed shortness of breath.

One sister suffered from diabetes mellitus. The patient had adequately treated persistently seropositive syphilis. Two stillbirths had occurred, the most recent being three months before admission.

The temperature was 99.6, pulse 110, respirations 36, blood pressure 120/80, and weight 247 lbs. The physical

examination showed an uncomfortable, mildly disoriented obese negro who lay panting in bed. She was severely dehydrated. There were intertrigo, vaginitis and evidence of chronic pelvic inflammatory disease. The examination was otherwise not remarkable.

The Kahn test was positive (4 Kahn Units), the urine positive for sugar (four plus) and acetone (three plus). The red blood count was 5,850,000, the hematocrit 49 per cent and the sedimentation rate (Westergren) 48 mm./hr. The white count was 11,550, with 74 per cent polymorphonuclear forms. The admission blood glucose level was 420 mg./100 ml., and the carbon dioxide combining power 13.9 vol. per cent (6.3 mM/L).

The acidosis was controlled, in a period of 24 hours, after the administration of a total of 810 units of regular insulin, 6500 ml. of normal saline and 550 gm. of glucose parenterally. After a period of observation in the hospital, she was discharged on an 820 calorie reducing diet, with 60 units of protamine zinc insulin daily. Over a period of three months, her insulin requirement gradually dropped so that, by May 1948, she was aglycosuric without insulin. During this time she lost 20 lbs. of weight.

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STUDIES OF THE DISTRIBUTION OF RADIOACTIVE IODINE IN THE PRESENCE OF SERUM AND URINE IN DIFFERENT LEVELS OF THYROID FUNCTION

There is prob-
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One of the earliest uses of radioiodine was as a tool for the study of thyroid physiology (1-7). Estimations of the relative quantities of isotope stored in the thyroid have been made by means of a Geiger-Müller tube held over the gland and by determining the amount present in the urine and blood. The rate with which the level of radioiodine changes in these compartments has also been studied (7, 8).

In subjects with euthyroidism, radioiodine was found (8) to be excreted rapidly, within a few hours after its administration, but a plateau tended to appear after 48 hours. In myxedematous individuals, excretion was slower initially but it was more persistent, requiring four days or more; the total amount excreted after several days was greater in myxedematous subjects than in normal or thyrotoxic ones. In untreated thyrotoxic patients the rate of excretion was less than in either of the other two groups. The curves describing excretion became asymptotic relatively quickly.

The differences in the metabolism of iodine in various functional states of the thyroid have prompted an investigation of the usefulness of radioiodine as a diagnostic test. One of the best tests not dealing with radioiodine that has been available is the determination of the protein-bound iodine of the plasma (9). This test is so long and difficult that it can be performed satisfactorily only under the direction of a few highly qualified individuals and even then some overlapping in the values for different degrees of thyroid function may be found (10). Moreover, the test is unreliable if the patient has recently received iodine in organic form and if highly scrupulous technique is not followed in the collection of the plasma as well as in the determination. It seemed to us that in some institutions it might be possible to use radioiodine in tests which would be more accurate,

simpler and more rapid. It appears logical to assume that the quantity of protein-bound radioiodine in the serum, after the administration of tracer doses, might indicate the relative rate of manufacture of the thyroid hormone and its release into the blood stream. Of course, the values obtained would represent only an approximate balance between these two factors and the quantity of the hormone stored in the various fluids and tissues of the body, including the thyroid; also between the amount metabolized and excreted. Nevertheless, with methods not using the radioiodine, not only do these difficulties in interpretation exist, but also it is not possible to determine the time required for the changes to occur. The latter aspect would seem to be a very important one.

METHODS

Two types of study were conducted following the administration of a tracer dose of radioiodine subcutaneously. In one, frequent specimens of blood and urine were collected during a 24-hour interval, for a determination of the proportion of isotope present. In the other study, which consisted of a larger number of patients, urine was saved for 24 hours and a single specimen of blood was taken at the end of this interval. I^{131} obtained from Clinton Laboratories, Oak Ridge, Tennessee, was used for the studies; no carrier was added.

Determination of the radioiodine in the specimens was by the methods previously described (11). Trichloroacetic acid was used for precipitation of protein-bound isotope. The "DI" (diiodotyrosine iodide) fraction was separated from the "TI" (thyroxine iodide) by butyl alcohol precipitation (9).

In the first experiment there were seven individuals with euthyroidism, four with thyrotoxicosis, three with athyreosis and one with Addison's disease. The patients with thyrotoxicosis had had no treatment. Those with athyreosis were in a state of euthyroidism as a result of therapy with desiccated thyroid, thereby affording an opportunity to observe the effects of the thyroid upon

certain aspects of iodine metabolism. Results obtained in patients so treated would be expected to be unlike those of untreated patients in that the metabolic rate would be higher in the former group, but the two types are similar in that in both instances essentially no thyroxin can be synthesized by the thyroid. The subject with Addison's disease was adequately treated with desoxycorticosterone. Each of the patients in this experiment was hospitalized while the studies were conducted.

In the second experiment there were 20 subjects with euthyroidism, eight with athyreosis receiving treatment with desiccated thyroid, 17 with definite thyrotoxicosis, and seven with questionable thyrotoxicosis. The group with euthyroidism consisted of ten internes, two nurses, and eight patients with a variety of non-thyroid diseases, none of which were severe. Ten of the thyrotoxic patients had had no previous therapy; the others had had propylthiouracil but none for four days or more preceding the test, and it was found that the results were similar to those without previous therapy. Two of the seven subjects with questionable thyrotoxicosis had been treated previously with propylthiouracil, but had not received any

for more than one week preceding the radioiodine. Each of the seven patients had a goiter. Very few of the subjects in this experiment were hospitalized.

RESULTS

The results of the first experiment are shown in Figure 1. The subjects with euthyroidism and the ones with athyreosis excreted radioiodine in the urine much more rapidly than did the patients with the thyrotoxicosis. None of the latter group excreted as much as 20 per cent of the injected dose within the first 24 hours, whereas each of the other two groups excreted distinctly more than this quantity. The myxedematous subjects had excreted essentially the same quantity of isotope as normals by the end of four hours. In the patient with Addison's disease, the excretion of isotope in the urine and its concentration in the

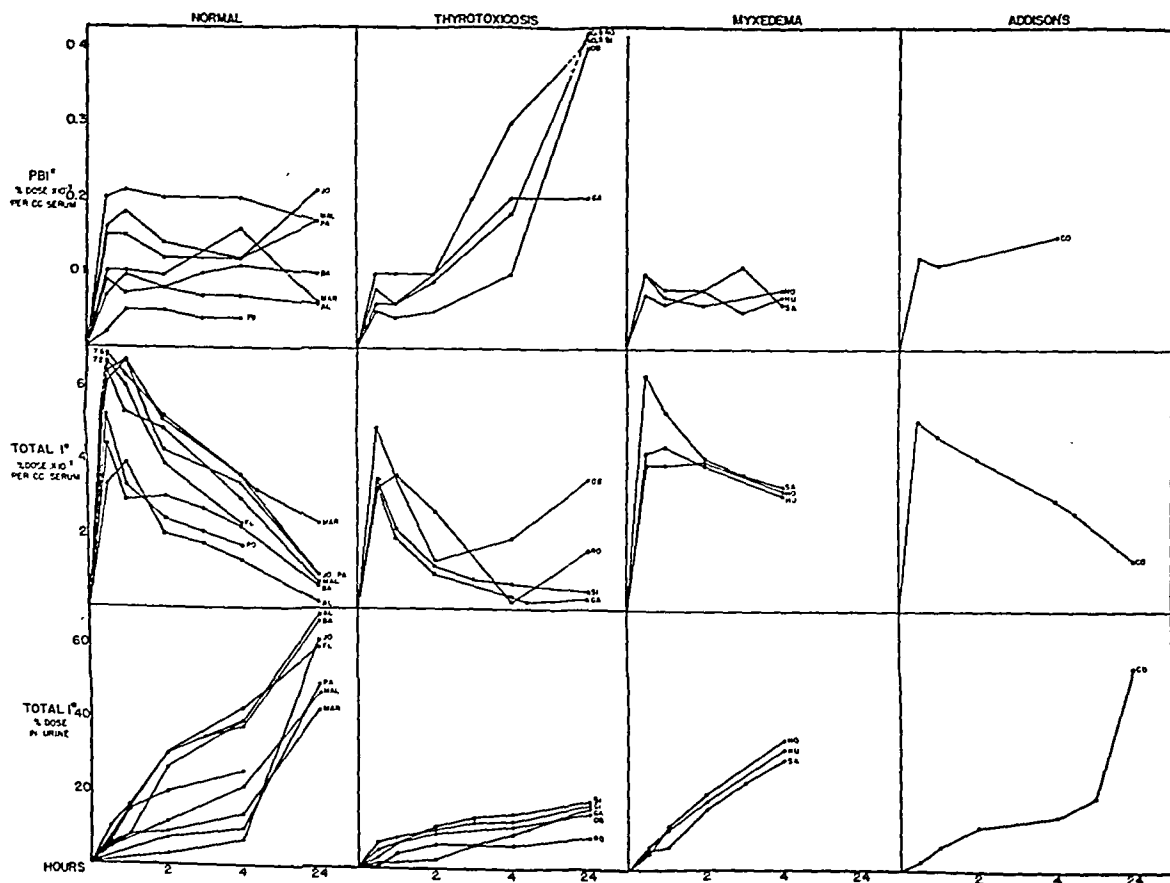


FIG. 1

Each patient was given 100 microcuries of radioiodine (I¹³¹) subcutaneously without any carrier. PBI* indicates the protein-bound radioiodine. All values are expressed with reference to the dose of isotope administered.

serum were within normal range. During the first four hours the concentration of total radioiodine in the serum of thyrotoxic patients tended to be lower than in the subjects with euthyroidism or

athyreosis. It can be observed in Figure 1 that two of the thyrotoxic patients had a second rise in the concentration of isotope. The quantity of protein-bound radioiodine rapidly reached a pla-

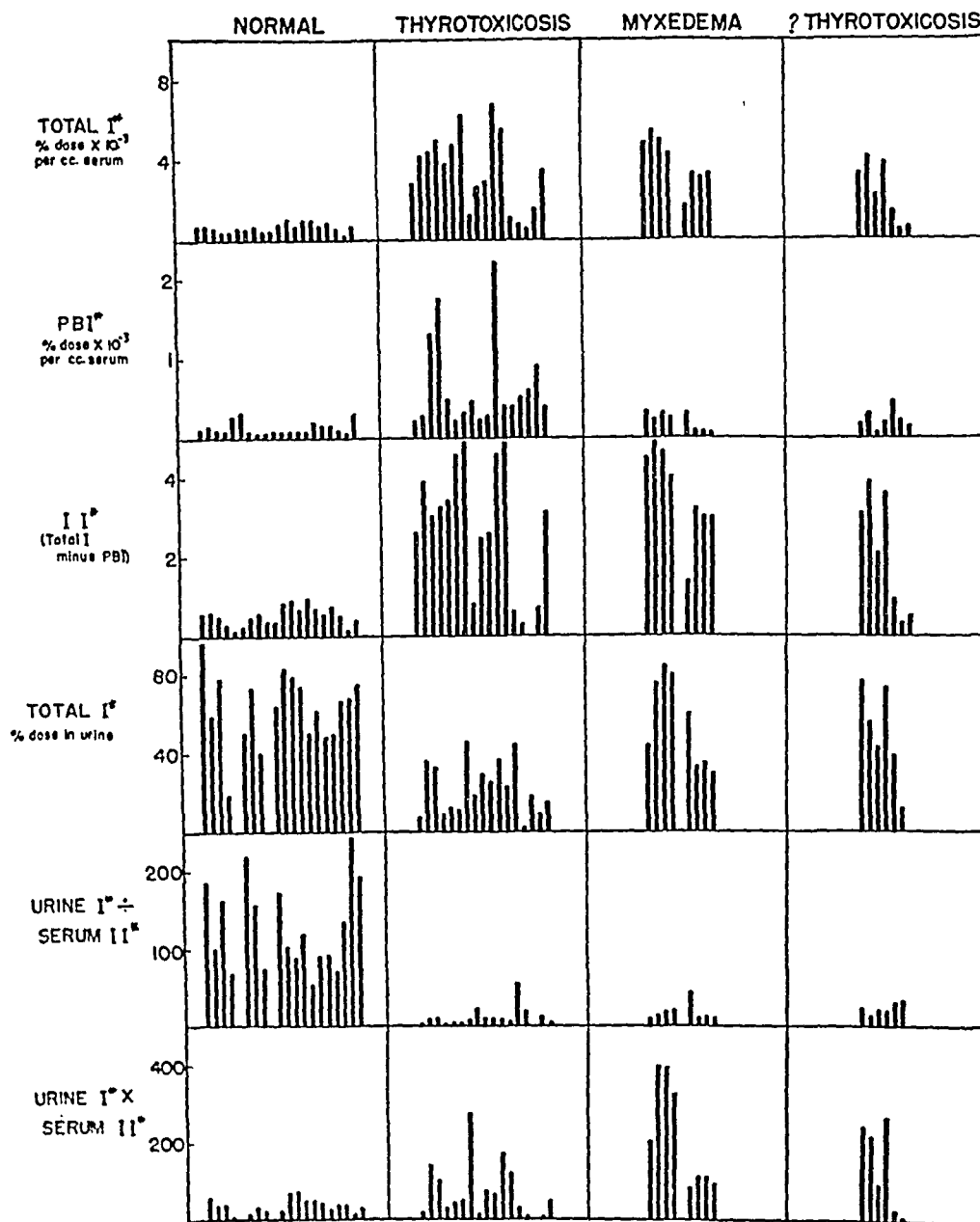


FIG. 2

The serum specimens were obtained 24 hours following the administration of radioiodine; during this interval all urine was saved for analysis; two patients neglected to collect urine. The quantity of radioiodine in inorganic (II*) form in the serum was derived by subtracting the protein-bound radioiodine (PBI*) from the total radioiodine content of serum. The reasons for empirically dividing and multiplying the values for the radioiodine in urine by the inorganic radioiodine in the serum are discussed in the text. The data shown in the seventh column of the group with "? thyrotoxicosis" are those of a patient who was subsequently shown not to have thyrotoxicity. Unfortunately, this patient failed to save all of his urine. The diagnosis of the individual with data presented in the sixth column is still questionable. In four patients myxedema was primary and in the other four (last four columns) it was secondary.

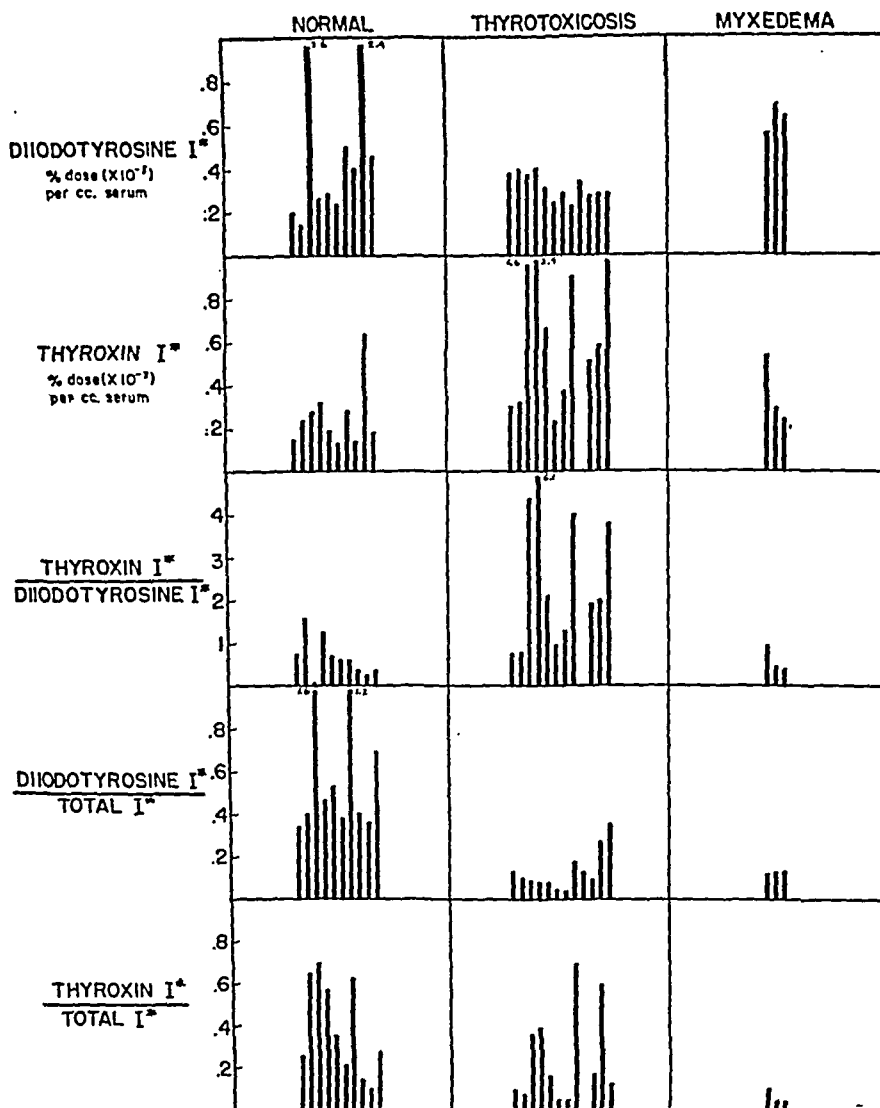


FIG. 3

These data were collected upon some of the patients represented in Figure 2.

teau in the individuals with euthyroidism and athyreosis. In the ones with thyrotoxicosis the increase was slow in the first few hours, but after 24 hours it was markedly higher in three of the four thyrotoxics than in any other subject. These observations prompted the performance of the second experiment, the results of which are shown in Figure 2.

The concentration in the serum of total radioiodine was distinctly greater in the patients with thyrotoxicosis than it was in normal individuals. In considering these findings in conjunction with the subnormal excretion of radioiodine in the urine within a 24-hour interval, it must be borne in

mind, as illustrated in Figure 1, that during several hours the concentration of total radioiodine in the serum was less in the thyrotoxic subjects than in normals. Moreover, in the former group, during the latter part of the 24-hour interval more of the radioiodine was protein-bound and thus less available for excretion.

The concentration of total and inorganic radioiodine serum of myxedematous patients was greater than normal, while the protein-bound radioiodine of the serum and the quantity of isotope in the urine were essentially normal. As to why there should be the discrepancies in the ratios of inorganic iodine in the serum and the quantity in

the urine, the answer is not clear. However, we attempted to emphasize these discrepancies in the different groups by empirically dividing the urine values by the serum inorganic iodine, and, also by multiplying them. As seen in Figure 2, there is a marked difference in the ratio of urine to serum values in the normal group as compared to the others. The product of the values is distinctly greater than normal in each of the subjects with athyreosis; it is also greater in some, but not all, of the thyrotoxic patients.

In serum of some of the patients the diiodotyrosine radioiodine and thyroxin radioiodine fractions were determined (Figure 3). No very striking difference in these fractions was observed in the various groups. The ratio of thyroxin to diiodotyrosine tended to be somewhat greater in the thyrotoxic patients, but there is too much overlapping of values to be of practical significance. On the other hand, the ratio of diiodotyrosine radioiodine to total radioiodine is definitely greater in the normal subjects than in the others. The tendency in normals for a greater ratio of thyroxin radioiodine to total radioiodine was an unexpected finding.

DISCUSSION

All of the indirect methods that have been proposed for the evaluation of the functional state of the thyroid gland by the determination of its uptake of radioiodine are associated with certain inaccuracies. Among these are the variations in thickness and contour of the neck, innumerable factors affecting kidney function, differences in the volume of the fluid compartments, and probably the rate of metabolism of the thyroid hormone and of its synthesis and concentration in non-thyroid tissues. Although Hertz, Hamilton and Soley and their colleagues (1-5), respectively, found that patients with hyperthyroidism accumulated a larger proportion of the tracer dose in the thyroid than did normal subjects, Astwood and Stanley (12) found that the concentration gradients of the group with thyrotoxicosis was not significantly above the normal range. The failure of the latter investigators to administer any carrier iodine might account for some of the difference in results. Whereas some investigators (3, 4) have measured the excretion of radioiodine in the urine as an index to the quantity retained in the thyroid gland, a

poor correlation has been found (13) between determinations of this type and direct measurements over the thyroid. However, all of the other clinical tests of thyroid function are beset with difficulties.

On the basis of data which we have presented, as well as those of others, determinations of the proportion of a tracer dose excreted in the urine are of diagnostic importance, but are not infallible. The measurements of the total radioiodine concentration in the serum, or of the measurements of the protein-bound thyroxin and diiodotyrosine radioiodine components did not demonstrate as clear a differentiation of the various levels of thyroid function as was desired. Nevertheless, it is believed that these estimations and certain ones of the ratios, that have been discussed, may be of some significance. It seems likely that greater differentiations in the protein-bound radioiodine components could be demonstrated if longer intervals were permitted to elapse before collecting the specimens, or by following the rate of the changes.

SUMMARY

Studies were conducted using radioiodine for the evaluation of different degrees of thyroid function, as found in subjects with thyrotoxicosis, athyreosis, or euthyroidism. Following the administration of 100 microcuries of radioiodine (I^{131}) carrier free, it was confirmed that thyrotoxic patients excreted less of the isotope in the urine than did the other groups. Although the concentration in the serum of total radioiodine tended to be less in the thyrotoxic group than in the others, within the first few hours, after 24 hours the total radioiodine and its protein-bound, thyroxin, and inorganic radioiodine components tended to be super-normal. In the group with athyreosis the total and inorganic radioiodine concentrations were super-normal. The ratio of diiodotyrosine radioiodine to thyroxin radioiodine, as well as a somewhat empirically calculated ratio of urine radioiodine and serum inorganic radioiodine, was distinctly less in the patients with thyrotoxicosis or athyreosis than in the normals. The product of the radioiodine of the urine and of the inorganic form of the serum was distinctly greater in the subjects with athyreosis than in normals and tended to be

greater in the former group than in the ones with thyrotoxicosis.

There was no good correlation of the results with the various degrees of thyrotoxicity.

Tests of the type reported, like all of the others of thyroid function, should be considered in conjunction with the clinical findings.

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EFFECTS OF ADRENOCORTICOTROPHIC HORMONE ON NEUROMUSCULAR FUNCTION IN PATIENTS WITH MYASTHENIA GRAVIS¹

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The adrenocorticotrophic hormone of the pituitary gland has been administered to patients with myasthenia gravis mainly on the basis of the following observations and inferences: (1) the immediate cause of the symptoms of myasthenia gravis is a decrease of acetylcholine synthesis (1-4); (2) administration of the adrenocorticotrophic hormone increases acetylcholine synthesis *in vivo* (5); (3) increase of the lymphatic tissue (round-cell infiltration of various organs, mainly striated muscle [6]) and "hyperfunctioning" thymus (7) have been found in patients with myasthenia gravis. Tissue fractionation studies (8, 9) have shown that one of the sources of the substances that inhibit acetylcholine synthesis is the thymus. Administration of the adrenocorticotrophic hormone induces reduction in the mass of the thymus and the lymphatic tissue (10, 11); (4) removal of the pituitary gland in rats induces changes in the electromyogram (12) that closely resemble the abnormalities noted in patients with myasthenia gravis (13, 14); (5) the pituitary gland of several patients who died of myasthenia gravis showed accumulation of an eosinophilic colloid material suggesting altered function of the gland (15-19).

This report aims to illuminate the nature of myasthenia gravis by a further analysis of its phenomenology. Therapeutic implications are outside its scope.

MATERIAL

The effect of the administration of adrenocorticotrophic hormone of the pituitary gland was studied in five patients moderately to severely ill with myasthenia gravis.²

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²Since the completion of this manuscript three additional patients with myasthenia gravis have been given

Patient R. G., a 45 year old woman, had had myasthenia gravis for ten years. She received a total of 180 mg. of neostigmine bromide a day, distributed over the waking hours, taken in three hourly intervals, 25 mg. of ephedrine sulfate once a day, and 1 gram of potassium chloride three times a day. While on this medication, she was able to rise from her bed and to sit by it in an armchair. She was barely able to walk very short distances when aided by an attendant. She had ptosis of the left eye-lid; her extraocular movements were limited in all directions. She also exhibited marked weakness and easy fatigability of the muscles of the palate, tongue, deglutition, chewing, face, and extremities. In addition, she had severe anorexia.

Patient H. L., a 24 year old woman, had had myasthenia gravis for four years. She received a total of 300 mg. of neostigmine bromide a day, distributed over the waking hours, taken in three hourly intervals, 25 mg. of ephedrine sulfate three times a day, 1 gram of potassium chloride three times a day, and 0.13 gram of guanidine hydrochloride three times a day. When on this medication, she was able to walk but showed severe general weakness, ptosis of the right eye-lid, and serious difficulty in swallowing. She had marked muscle weakness and easy fatigability of the muscles of the palate, tongue, deglutition, chewing, face, and extremities. There were periods when general weakness was much intensified and walking became almost impossible. She had anorexia.

Patient M. Y., a 31 year old woman, had had myasthenia gravis for nine years. Removal of the thymus in 1942 was followed by only a temporary remission. The patient received the total amount of 150 mg. of neostigmine bromide a day, distributed over the waking hours, taken in three hourly intervals. No other medication was taken. When on this medication, she was able to walk and do light housework. She exhibited general muscle weakness and easy fatigability, ptosis of one eye-lid, marked weakness of the muscles of the tongue, deglutition, chewing, palate, face, and arms. When speaking she held up her chin with her hand. She had anorexia.

adrenocorticotrophic hormone in the manner described above. The results support in general the inferences of this study.

Patient A. S., a 37 year old woman, had had myasthenia gravis for 13 years. She received a total of 112 mg. of neostigmine bromide a day, distributed over the waking hours, taken in three hourly intervals. No other medication was taken. When on this medication she was able to walk and to do light housework. She exhibited marked general muscle weakness and easy fatigability. She had ptosis of the left eye-lid. She also exhibited weakness of the muscles of the palate, tongue, deglutition, face, arms, and mainly the legs. She had moderate anorexia.

Patient J. R., a 29 year old woman, had had myasthenia gravis for 17 years. She received a total of 45 mg. of neostigmine bromide a day, distributed over the waking hours, taken in six hourly intervals, and 25 mg. of ephedrine sulfate. When on this medication she was able to walk and to do light housework. She exhibited weakness of her leg muscles, moderate weakness of the muscles of the face, and ptosis of the left eye-lid.

During the three years before this special study began the patients experienced minor transient fluctuations but no long lasting or significant changes in their clinical states.

METHOD

The five patients were permitted to take known amounts of neostigmine bromide, and, if already receiving it, other medications. Throughout the study the patients were encouraged in any spontaneous attempts to reduce the amounts of the medications taken. The patients were warned against expecting improvement of their symptoms as a result of the injections received.

Upon admission to the hospital, electromyographic and myographic studies were performed once a day approximately at the same time of the day. After a one week period of observation each patient received by intramuscular injections 20 mg. of the adrenocorticotrophic hormone (Armour) every six hours for five days. Tests were performed during the administration of the hormone and afterwards until the writing of this report (approximately three months).

Controls. Ten healthy women aged from 27 to 44 years served as controls.

Electromyography

Records of muscle action potential were taken from healthy women and from patients with myasthenia gravis by the following method (6, 7): The forearm was firmly fixed in the supine position onto a padded board by means of broad straps placed across the arm near the elbow and the wrist. Of the two silver recording electrodes (0.5 sq. cm.), one was fixed with adhesive tape to the skin over the ventral surface of the first phalanx of the fifth finger and the other over the surface of the hypothenar eminence at a distance of about 5 cm. from the first electrode. The electrodes were connected to the grid terminals of an amplifier feeding into an oscilloscope. The stimulating electrodes consisted of two silver plates, the larger being affixed to the skin over the triceps

muscle and the smaller, a movable electrode 0.5 cm. in diameter, being pressed firmly against the skin over the ulnar nerve just above the elbow. Good contact was established by the use of electrode jelly over the skin, which was partially deprived of its superficial epidermis. Ten and 30 pulses per second, each of 100 microseconds' duration and of "supramaximal" intensity, were administered for two minutes. The sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and muscle action potentials were superimposed on the screen of the cathode ray tube. The action potential was recorded photographically.

Electromyograms were recorded for seven days before administration of the hormone, the five days during administration of the hormone, four days after completion of the series of administrations of the hormone, and at biweekly intervals thereafter. The records were taken at the same time of the day, three hours after the administration of neostigmine bromide before and during administration of the hormone, and from six to 15 hours after administration of neostigmine bromide after completion of the series of administrations of the hormone.

Myography

Muscle function was also tested by an ergograph. The ergograph consisted of a heavy spring attached to an isotonic lever writing on a kymograph. The spring was stretched 2.5 cm. once a second by the index and middle finger of the right hand exerting a tension of 15 kg. until fatigue occurred. The patients with myasthenia gravis performed the test once a day for a week before the period of administration of the adrenocorticotrophic hormone, during the five days of administration, and for four days after completion of administration of the hormone. Thereafter, the test was performed biweekly. The myograms were recorded immediately after completion of the electromyograms.

Biochemical studies (Acetylcholine Synthesis)

Acetylcholine synthesis in the presence of serum was studied following the method described by Torda and Wolff (2) before the injection period of the hormone, the third day after completion of the injection period, and biweekly thereafter. The method consists of incubation of a tissue containing choline acetylase with blood serum and determination of the amount of acetylcholine formed during the period of incubation.

RESULTS

General observations

During the five days of administration of the adrenocorticotrophic hormone the patients experienced a gradually increasing disability lasting until the second day after the end of the series of injections. This disability consisted of malaise, headache or "heaviness of the head," disturbance

in the menstrual cycle (bleeding), slightly increased fatigability, occasional diarrhea, abdominal cramps, and complaints of hot feet and hands.

On the second or third day after the last injection of the adrenocorticotrophic hormone the situation changed: the patients exhibited and described increasing well being and began to reduce the daily intake of neostigmine bromide and the other agents. Patient H. L. reduced the total of 300 mg. of neostigmine bromide a day to 45 mg. and omitted the other medications; patient M. Y. reduced the total daily intake of neostigmine bromide from 150 mg. to 15 mg.; patient A. S. reduced the total daily intake of neostigmine bromide from 112 mg. to 22.5 mg.; patient R. G. reduced the total daily intake of neostigmine bromide from 180 mg. to 90 mg.; and patient J. R. reduced the total daily intake of neostigmine bromide from 45 mg. to 15 mg. Also, more visible and measurable changes occurred in the patients that might indicate the beginnings of a partial remission. Thus, there was a clearly evident improvement of the facial muscle strength, the general muscle weakness and easy fatigability diminished, and the ptosis of the eye-lids was less evident. Difficulty in swallowing diminished significantly and the patients were able to hold heavy objects in their hands for a period of time. The appetite for food increased. However, in all instances the remission was incomplete. The muscle groups most severely involved (*i.e.* extraocular muscles and leg muscles in patient R. G., muscles of the forearm and foot in patient H. L., some muscles involved in chewing in patient M. Y., leg muscles in patients A. S. and J. R.) showed only partial recovery of function. This improved neuro-muscular function persisted from the completion of administration of the adrenocorticotrophic hormone to the writing of this report (approximately three months).

Electromyography

Healthy subjects maintained the muscle action potential during indirect stimulation with ten pulses per second for two minutes unaltered (13, 14) (Figure 1A). The action potential decreased on the average 12 per cent during indirect stimulation with 30 pulses per second for two minutes.

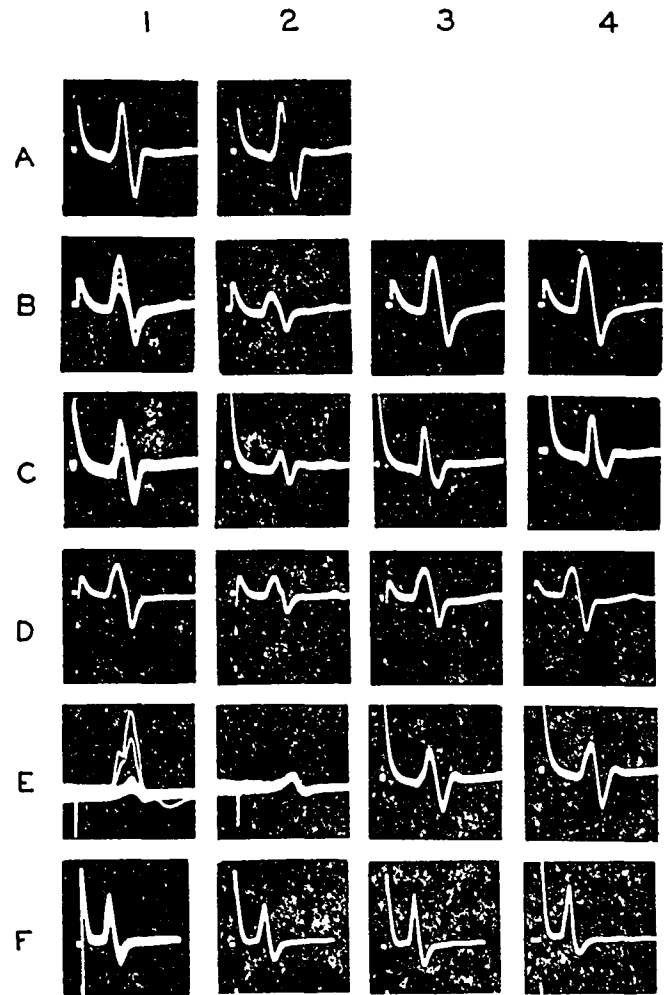


FIG. 1. EFFECT OF ADRENOCORTICOTROPHIC HORMONE ON MUSCLE ACTION POTENTIAL DURING REPETITIVE INDIRECT STIMULATION WITH TEN PULSES PER SECOND (PERCUTANEOUS STIMULATION OF THE ULNAR NERVE)

A. Muscle action potential of a control subject:

1. Taken during the first few pulses of a 30 second stimulation period;
2. Taken at the end of the 30 second stimulation period; the action potential was maintained unchanged.

B. Muscle action potential of patient H. L.:

1. Taken during the first few pulses of a 30 second stimulation period before administration of the hormone;
2. Taken at the end of the 30 second stimulation period; the action potential decreased during the first few pulses of stimulation and was maintained at the new level thereafter;
3. Taken during the first few pulses of a 30 second stimulation period the third day after completion of the series of administrations of the hormone;
4. Taken at the end of the 30 second stimulation period; the action potential was maintained unaltered.

C. Muscle action potential of patient M. Y.:

1. Taken during the first few pulses of a 30 second

stimulation period before administration of the hormone;

2. Taken at the end of the 30 second stimulation period; the action potential decreased during the first few pulses of stimulation and was maintained at the new level thereafter;
3. Taken during the first few pulses of a 30 second stimulation period the third day after completion of the series of administrations of the hormone;
4. Taken at the end of the 30 second stimulation period; the action potential was maintained unaltered.

D. Muscle action potential of patient A. S.:

1. Taken during the first pulse of a 30 second stimulation period before administration of the hormone;
2. Taken at the end of the 30 second stimulation period; the action potential decreased during the first few pulses of stimulation and was maintained at the new level thereafter;
3. Taken during the first pulse of a 30 second stimulation period the third day after completion of the series of administrations of the hormone;
4. Taken at the end of the 30 second stimulation period; the action potential was maintained unaltered.

E. Muscle action potential of patient R. G.:

1. Taken during the first few pulses of a 30 second stimulation period before administration of the hormone;
2. Taken at the end of the 30 second stimulation period; the action potential decreased during the first few pulses of stimulation and was maintained at the new level thereafter;
3. Taken during the first few pulses of a 30 second stimulation period three months after completion of the series of administrations of the hormone;
4. Taken at the end of the 30 second stimulation period; the action potential remained unaltered.

F. Muscle action potential of patient J. R.:

1. Taken during the first few pulses of a 30 second stimulation period before administration of the hormone;
2. Taken at the end of the 30 second stimulation period; the action potential was maintained within normal limits.
3. Taken during the first few pulses of a 30 second stimulation period the third day after completion of the series of administrations of the hormone;
4. Taken at the end of the 30 second stimulation period; the action potential remained unaltered.

Since the recording electrodes were not moved during the 30 second stimulation period the action potentials of columns 1 and 2 and columns 3 and 4 are comparable. Since the recording electrodes could not be placed in exactly the same position on successive days the action

potentials in column 1 are not comparable with those in column 3.

In all records the sweep circuit of the oscilloscope was synchronized with the stimulator so that successive stimuli and muscle action potentials were superimposed on the screen of the cathode ray tube.

In patients severely ill with myasthenia gravis the muscle action potential in affected muscles rapidly decreased during repetitive indirect stimulation (13, 14). Thus, before administration of the adrenocorticotrophic hormone, the action potential of patients H. L., M. Y., R. G., and A. S. decreased during the first few seconds of stimulation and was maintained at the new amplitude during continuation of the stimulation for two minutes. There was a considerable daily variation in the decrease in the amplitude of the muscle action potential. The decrease of the action potential was, however, in all instances over 35 per cent with 10 pulses per second and over 55 per cent with 30 pulses per second (Figure 1B-1F). The muscle action potential in patient J. R. was similar to the action potential of healthy controls, as is always the case in patients moderately or mildly ill with myasthenia gravis (13).

After completion of administration of the adrenocorticotrophic hormone and while on reduced medication the patients maintained the action potential during repetitive indirect stimulation with 10 and 30 pulses per second in a manner approximating that of healthy subjects (Figure 1B-1F). At the time of writing this report, approximately three months after completion of the administration of the hormone, the action potential was still maintained in a manner approximating that of healthy subjects.

Myography

The patients were trained for a few days in the use of the ergograph during the preliminary period of observation. The amount of work performed varied daily. The average number of contractions were, however, far below the average number of contractions of control subjects, except with patient J. R. The average number of contractions before occurrence of fatigue was 31 in patient R. G., 44 in patient A. S., 47 in patient M. Y., 75 in patient H. L. and 110 in patient J. R. The average of the ten healthy controls was 150.

During the period of administration of the adrenocorticotrophic hormone the number of contractions slightly and gradually increased. After completion of administration of the adrenocorticotrophic hormone the work performance continued to increase. The number of contractions increased from the first to the fifth day after completion of the series of administrations of the hormone. The average number of contractions during the first four days after completion of administration of the hormone was 98 in patient R. G., 120 in patient A. S., 165 in patient M. Y., 233 in patient H. L., and 160 in patient J. R. The increase in performance was maintained at the time of writing of the present report (approximately three months) (Table I). Although this test in-

volves motivation, a sudden and dramatic increase in performance suggests an improvement of the function of the neuro-muscular system per se.

Biochemical studies (Acetylcholine synthesis)

The synthesis of acetylcholine in the presence of body fluids (1-3) from patients with myasthenia gravis is decreased, suggesting that the body fluids contain in excess substances that inhibit the activity of choline acetylase. The more severe the myasthenia gravis the less well the blood serum supports the activity of choline acetylase.

In the presence of blood serum from patients with myasthenia gravis before administration of the adrenocorticotrophic hormone the synthesis of acetylcholine decreased 25 to 55 per cent (Table II). In the presence of blood serum taken the third day following completion of the series of administrations of the hormone and biweekly thereafter until writing of the present report (approximately three months) the synthesis of acetylcholine greatly increased and became similar to that occurring in the presence of serum from healthy subjects (Table II). The amount of acetylcholine synthesized in the presence of serum from the controls averaged $2.08 \mu\text{g} \pm 7$ per cent per 100 mg. tissue containing choline acetylase.

GENERAL COMMENT

Administration of the adrenocorticotrophic hormone of the pituitary gland to patients with myasthenia gravis was first begun in 1944 by Torda and Wolff (5, 20). A partial remission consisting essentially in the ability to perform more work while taking significantly reduced amounts of medication occurred in the two patients with myasthenia gravis treated with the hormone. Because satisfactory objective procedures testing neuro-muscular function per se had not been elaborated in this laboratory at the time and since spontaneous remissions and improved motivation make evaluation of clinical changes difficult, inferences concerning the effect of the adrenocorticotrophic hormone in patients with myasthenia gravis were deferred.

In 1948 Soffer and collaborators (21) using no objective testing procedures of the neuro-muscular function reported a remission of the symptoms of

TABLE I
Effect of adrenocorticotrophic hormone on work performance (ergograph)

Treatment	Time	Number of contractions before occurrence of fatigue					
		Patients with myasthenia gravis					Healthy women
		R. G.	A. S.	M. Y.	H. L.	J. R.	
Before administration of the hormone		28	49	30	58	126	130
		23	40	53	77	100	150
		26	35	56	74	102	170
		34	34	43	83	86	184
		39	32	53	79	92	120
		35	56				110
		33	54				170
		40					130
		31	38				173
							167
During administration of the hormone	First day	42	58	53	99	92	
		50	42	46		104	
	Second day	53	55	60	103	108	
		57	45	67	101	110	
	Third day	72			109	120	
		76			111		
	Fourth day	73	63	74			
		70	60	50			
	Fifth day	90	61	93	120	140	
		100	54	50	120	150	
After administration of the hormone	First day	89	80	90	200	141	
		80	80				
	Second day	90	120	165	232	150	
		93	124	170			
	Third day	105	140	186	245	174	
		109	130	180			
	Fourth day	124	135	190	255	170	
		98	130	150			
	Two weeks	103	137	185	250	180	
		103					
	Four weeks	110		192	240		
	Eight weeks	112		199	245		
	12 weeks	106		187	240		

myasthenia gravis in a moderately ill woman after administration of the adrenocorticotrophic hormone.

Hellman (22) reported an augmentation of the symptoms of patients with myasthenia gravis as a result of administration of the adrenocorticotrophic hormone. However, this impairment seems to have occurred during the period of injection with the hormone, an effect also noticed in the patients of the present study. Decrease in symptoms in the latter patients was evident only after the second day following the last injection of the hormone.

Increased general disability during the period of administration of the adrenocorticotrophic hormone may result from the sudden changes in many phases of metabolism. Some of these are a decrease of the glutathione content of blood (23, 24), a sudden increase of the secretion of many steroid hormones (23-28), an adverse effect on carbohydrate metabolism, and disturbances in the

remission, although incomplete, was sudden in onset, was significant and long lasting, encouraging the inference that administration of the hormone and the occurrence of the remission were causally related. Remission must be, by the nature of the disorder, incomplete, since after patients with myasthenia gravis have had muscle dysfunction for some years, as was the case in the patients of this study, irreversible structural changes in muscle probably occur. Even assuming that regeneration under suitable conditions may ultimately take place, such regeneration would not exhibit itself in a short time.

If the view suggested by this laboratory be valid, *i.e.*, that the immediate cause of the symptoms of patients with myasthenia gravis is a decrease in the synthesis of acetylcholine (1-4) then the observation that administration of the adrenocorticotrophic hormone increases the synthesis of acetylcholine (5) becomes extremely pertinent to an understanding of the apparent remission of symptoms observed in patients with myasthenia gravis after administration of the adrenocorticotrophic hormone.

TABLE II

Effect of adrenocorticotrophic hormone on the ability of blood serum to support acetylcholine synthesis

Experiment	Amount of acetylcholine synthesized in per cent of control				
	Patients				
	R. G.	H. L.	M. Y.	A. S.	J. R.
Before administration of the hormone	45	50	58	60	75
Third day after administration of the hormone	92	98	97	100	99
Two weeks after administration of the hormone	94	96	98	97	96
Three months after administration of the hormone	92	95	96		

electrolyte balance within the body (26). All of these changes impede the synthesis of acetylcholine (20, 29-31) and augment the symptoms of patients with myasthenia gravis (20). A decrease of symptoms in patients with myasthenia gravis studied at the New York Hospital in 1945 followed an experimentally induced (20) increase of the "reduced-glutathione" content of the body. The remission observed after administration of the adrenocorticotrophic hormone began a few days after the completion of the injections. The

SUMMARY

1. The adrenocorticotrophic hormone of the pituitary gland has been administered to five patients moderately to severely ill with myasthenia gravis.

2. Before administration of the adrenocorticotrophic hormone the patients exhibited the known decline of the amplitude of muscle action potential during repetitive indirect stimulation. Also, the amount of work performance on an ergograph averaged only 30 per cent of that performed by healthy subjects selected from the same sex and from similar age group as were the patients. The amount of acetylcholine synthesized in the presence of blood serum of the patients was below normal.

3. Four hundred mg. of the adrenocorticotrophic hormone were administered in 20 mg. amounts every six hours. During the period of administration the patients experienced an increase in disability. The second day after completion of the series of injections the patients experienced changes suggesting the beginnings of

an incomplete remission of the disorder. This consisted of marked improvement of muscle function while on appreciably reduced neostigmine bromide. The much reduced ability of blood serum to support acetylcholine synthesis returned to normal. Furthermore, the electromyogram and myogram became similar to those of healthy subjects. This incomplete remission precipitated by the adrenocorticotrophic hormone persisted for an, as yet, undefined period.

ACKNOWLEDGMENT

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THE SERUM PROTEINS IN PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT. ELECTROPHORETIC STUDIES¹

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Alterations in the composition of the serum proteins in hepatic disease have been studied by numerous workers using the electrophoretic method (1-11). The present investigation was undertaken to determine the changes in the serum proteins in portal cirrhosis by serial electrophoretic analyses during the course of prolonged medical management.

METHODS

Electrophoretic runs were performed with standard apparatus and technique, the experimental details having been given in an earlier report (11). The tests of hepatic function were performed by the usual methods as previously described (11), alkaline phosphatase being reported in Bodansky units.

CASE MATERIAL

The ten cases of the present study were all chronic alcoholics considered to have proven portal cirrhosis. The diagnosis was confirmed histologically by Vim-Silverman needle biopsy of the liver in seven of the ten cases, and in one patient (R. M. S.) also by autopsy. In one case (P. F.) the cirrhotic liver was visualized by peritoneoscopy and subsequently during an umbilical herniorrhaphy, but tissue for biopsy was not obtained. In two clinically typical cases (J. H. and C. R.), needle biopsy was attempted, yielding minute amounts of fibrous tissue considered insufficient for histologic diagnosis.

The ten cases were divided into two groups.

Group I. Five patients with hepatic insufficiency and ascites were studied with serial electrophoretic analyses of the serum proteins and various tests of hepatic function during the course of medical management for periods ranging up to ten months.

The initial electrophoretic analysis designated as "Day 0" was carried out before or very early in the course of medical management. Although a few patients had re-

ceived some form of therapy elsewhere prior to study, with one exception none had followed an adequate dietary regime for any appreciable period.

Group II. Five patients were studied after sustained clinical recovery under medical management. These individuals had been severely ill with hepatic insufficiency; ascites requiring paracenteses had been present in four, and pronounced jaundice in one. With treatment they had clinically recovered and had remained asymptomatic for more than two years.

THERAPEUTIC REGIME

The basic therapeutic regime consisted of a diet high in protein, carbohydrate and calories, containing 120 to 150 grams of protein, 350 to 450 grams of carbohydrate, a total of 3000 to 3500 calories, supplemented by 4 to 6 grams of choline chloride per day. The intake of sodium was restricted to 500 mg. per day in the patients in Group I as long as clinical evidence of ascites remained. The dietary regime was rigidly enforced during the hospital stay; after discharge patients were strongly urged to adhere to the diet, and were closely followed with numerous clinic visits. Not more than one paracentesis was done on any patient during the study; mercurial diuretics were used infrequently.

Other measures such as infusions of plasma or human albumin were employed as noted below. Salt-poor, concentrated human albumin was given as a 25% solution by slow intravenous infusion.

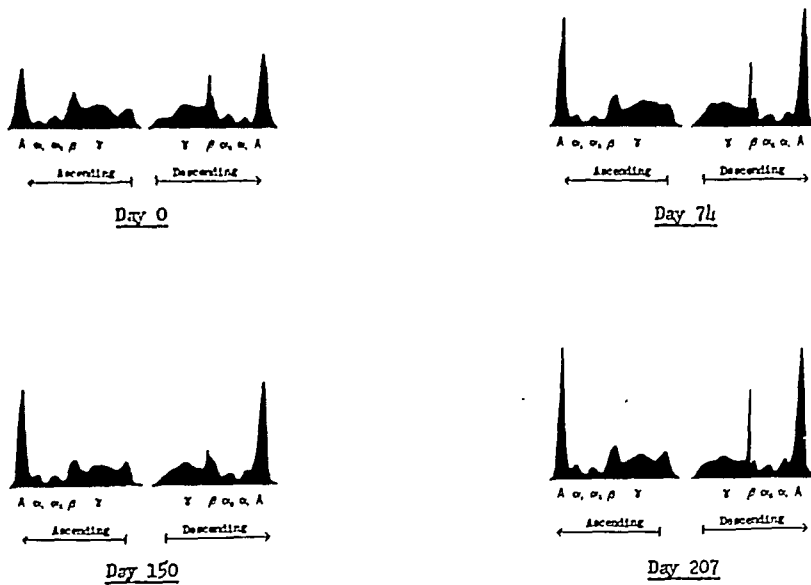
RESULTS

Group I. The initial electrophoretic analyses (Day 0) disclosed marked diminutions of the albumin fractions and elevations of the gamma-globulin fractions; the beta-globulins were somewhat less elevated (*cf.* normal serum, Figure 6 and Table VI).

J. K.—51 year old male. Hematemesis and melena occurred after an alcoholic debauch of two weeks' duration. On admission the patient had very severe jaundice, edema and ascites. Complete clinical recovery with subsidence of jaundice and disappearance of ascites occurred after three months of treatment. The patient remained free

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PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

J.K. - 51 year old male. Chronic alcoholic. Hematemesis and melena, extreme jaundice, ascites with paracentesis. Complete clinical recovery with disappearance of ascites on dietary regime.

FIG. 1. THE ALBUMIN PEAKS ARE INDICATED BY "A," AND THE GLOBULINS BY THEIR RESPECTIVE GREEK LETTER PREFIXES. THE UNLABELLED PEAKS ARE THE STATIONARY ANOMALOUS BOUNDARIES DUE TO GRADIENTS OF BUFFER SALT

of symptoms during almost five months of subsequent observation.

Serial electrophoretic analyses (Figure 1 and Table I) revealed pronounced evolution of the serum protein pattern toward normal. The tests of hepatic function also changed in the direction

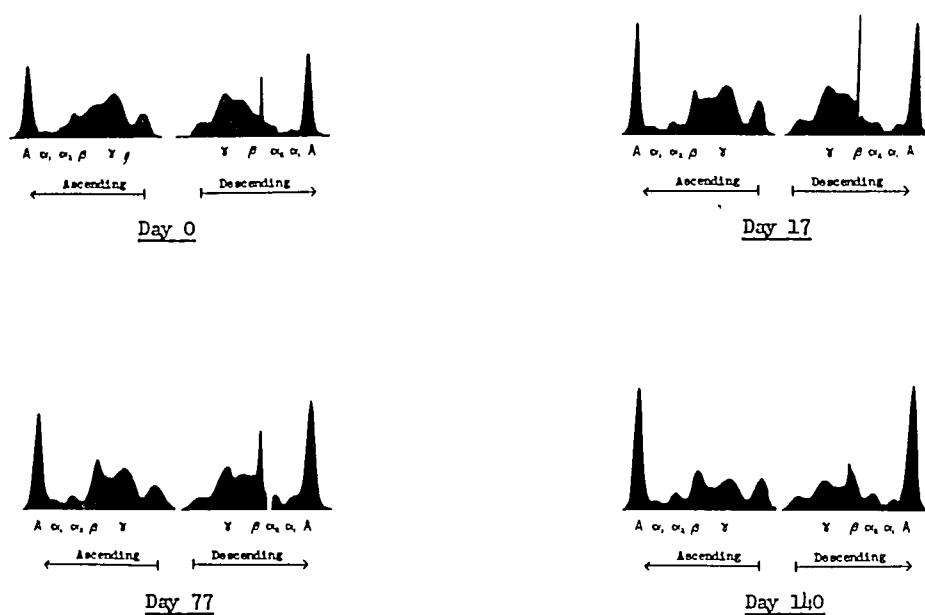
of normal. Despite complete clinical recovery, however, the final electrophoretic pattern on Day 207 was appreciably abnormal.

J.B.—62 year old male. Ascites with peripheral edema and pronounced jaundice had been present for approximately five months. The patient

TABLE I
PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

Sera of J.K.

	Electrophoretic Data											Howe sodium sulfate					bilirub		ceph	thym	alk phos	RSP %
	per cent					grams per cent					A/G	grams per cent										
	Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ		A/G	A/G	Alb	Glob	Tot	dir	tot				
Day 0	33.3	4.1	7.4	26.9	28.3	1.87	0.23	0.41	1.51	1.59	0.50	0.58	2.05	3.56	5.61	15.0	18.1	3+	turbid sera	17.8	--	
Day 74	40.5	5.5	6.6	18.5	28.9	2.77	0.38	0.45	1.26	1.97	0.68	0.86	3.16	3.67	6.83	0.9	1.9	3+		----	47	
Day 150	44.9	6.0	6.9	20.4	21.8	2.87	0.38	0.44	1.31	1.40	0.82	1.01	3.21	3.19	6.40	0.2	0.9	3+		5.2	--	
Day 207	45.6	6.6	5.8	19.4	22.6	2.87	0.42	0.37	1.22	1.42	0.84	1.27	3.53	2.77	6.30	0.4	1.2	1+		----	44	

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

J.B. - 62 year old male. Chronic alcoholic. Pronounced jaundice and ascites. Partial clinical improvement with disappearance of ascites on dietary regime to which patient did not adhere satisfactorily.

FIG. 2

improved only partially, and after discharge from the hospital did not adhere to the diet. The ascites disappeared in one month, but the jaundice persisted during the subsequent five months of observation.

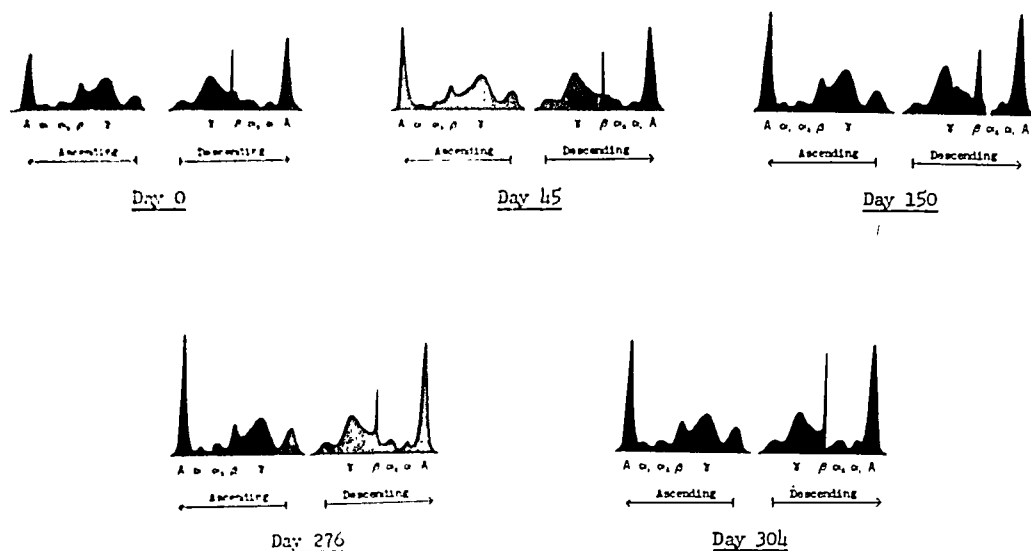
The electrophoretic patterns (Figure 2 and Table II) revealed pronounced evolution toward normal (fully as marked as in J. K.). The last pattern on *Day 140*, however, was appreciably abnormal.

M. S.—56 year old female. Jaundice had been present for six months; the patient was admitted to the hospital three weeks after the onset of ascites and edema of the ankles. On the dietary regime partial clinical recovery ensued during a period of five months, with subsidence of jaundice and ascites; the improvement was maintained during five additional months of observation, although the patient had not fully regained her usual vigor and sense of well-being.

TABLE II
PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

Sera of J.B.

	Electrophoretic data										Howe sodium sulfate					bilirub dir tot		ceph	thym	alk phos
	per cent					grams per cent					A/G	grams per cent								
	Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ		A/G	Alb	Glob	Tot					
Day 0	23.1	2.2	4.3	32.6	32.8	1.82	0.14	0.28	2.10	2.12	0.39	0.44	1.96	4.50	6.46	5.4	8.3	2+	10.7	5.4
Day 17	29.7	3.4	5.9	30.6	30.4	2.39	0.27	0.47	2.48	2.45	0.42	0.47	2.56	5.50	8.06	4.1	6.5	3+	12.1	8.5
Day 77	35.1	3.7	6.6	23.6	26.0	2.79	0.29	0.52	2.27	2.07	0.54	0.58	2.92	5.02	7.94	3.2	6.0	4+	16.8	9.4
Day 140	42.8	3.5	7.9	22.4	23.4	3.26	0.27	0.59	1.70	1.78	0.75	0.59	2.82	4.78	7.60	2.8	6.0	4+	15.2	10.2

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

M.S. - 56 year old female. Chronic alcoholic. Jaundice and ascites. Partial clinical recovery with disappearance of ascites on dietary regime.

FIG. 3

The serial electrophoretic patterns (Figure 3 and Table III) indicated a very gradual change in the direction of normal, but after the relatively asymptomatic period of five months, the pattern still remained markedly abnormal with a pronounced elevation of the gamma-globulin fraction (Day 304).

R. M. S.—43 year old male. Manifestations

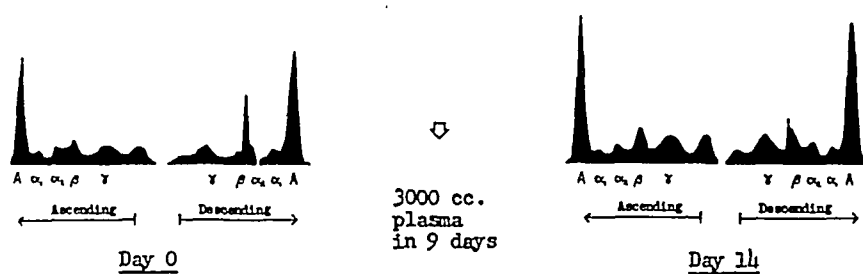
of cirrhosis had been present for more than two years with spider angiomas, episodes of jaundice, melena, edema and ascites necessitating paracentesis. Prior to admission, the patient had received elsewhere plasma, choline, and Vitamin K, as well as dietary treatment. The ascites disappeared after medical management including plasma infusions (3000 cc. in nine days). Just

TABLE III

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

Sera of M.S.

	Electrophoretic data											Howe sodium sulfate									
	per cent					grams per cent					A/G	grams per cent			bilirub dir tot		ceph	thym	alk phos	RSP %	
	Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ		A/G	Alb	Glob							Tot
Day 0	31.0	5.0	8.0	17.0	39.0	2.01	0.32	0.52	1.10	2.53	0.45	0.65	2.56	3.92	6.48	0.9	2.3	4+	7.4	7.5	99
Day 45	34.9	3.5	4.3	13.6	43.7	2.42	0.24	0.30	0.94	3.04	0.54	0.68	2.81	4.13	6.94	1.7	2.4	3+	10.7	9.2	110
Day 150	35.8	3.9	7.1	18.8	34.4	2.41	0.26	0.48	1.27	2.32	0.56	0.75	2.89	3.85	6.74	0.8	1.5	4+	6.8	12.9	--
Day 276	39.3	4.4	6.9	13.4	36.0	2.56	0.29	0.45	0.87	2.34	0.65	0.96	3.19	3.32	6.51	0.7	1.1	3+	7.2	12.0	92
Day 304	39.3	4.3	6.8	14.9	34.7	2.69	0.29	0.47	1.02	2.37	0.65	1.04	3.48	3.36	6.84	0.6	1.2	4+	8.2	12.3	--

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

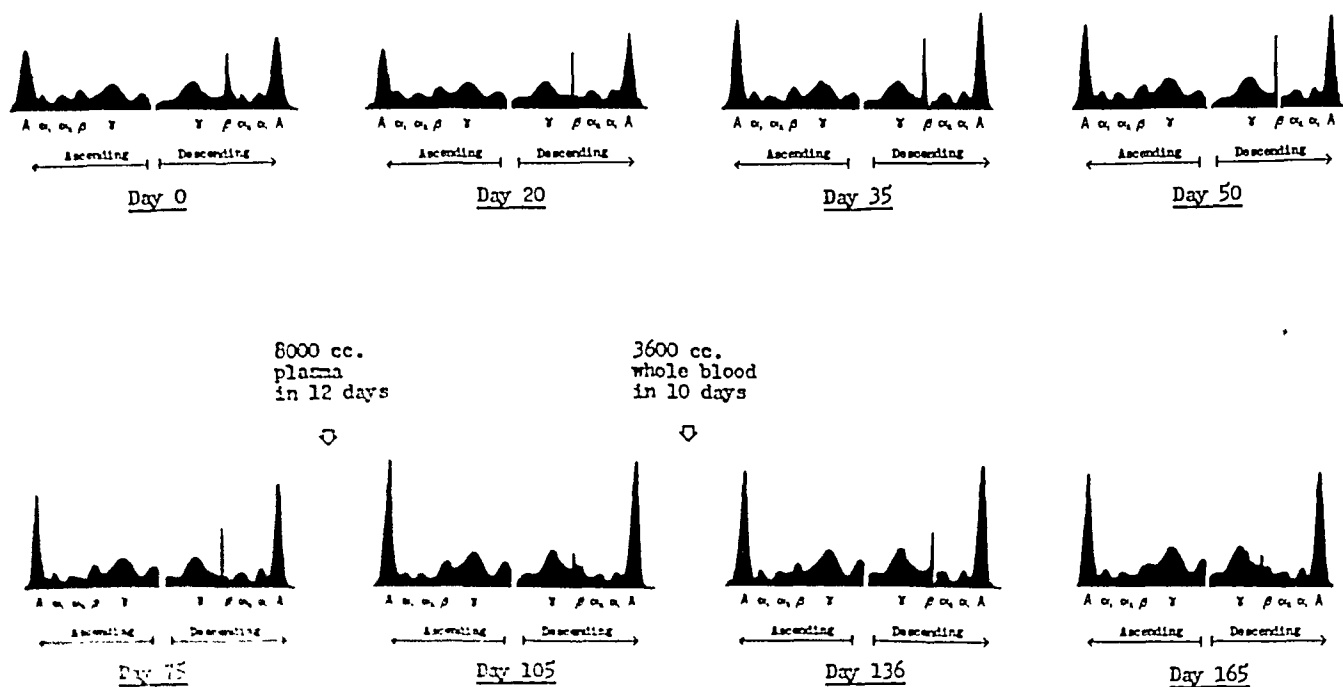
R.M.S. - 43 year old male. Chronic alcoholic. Melena, episodes of jaundice, ascites with paracentesis. Ascites disappeared after plasma infusions and dietary regime.

FIG. 4

prior to the intended date of discharge a massive hemorrhage from esophageal varices resulted fatally.

The alterations in serum protein composition were less pronounced in the initial electrophoretic

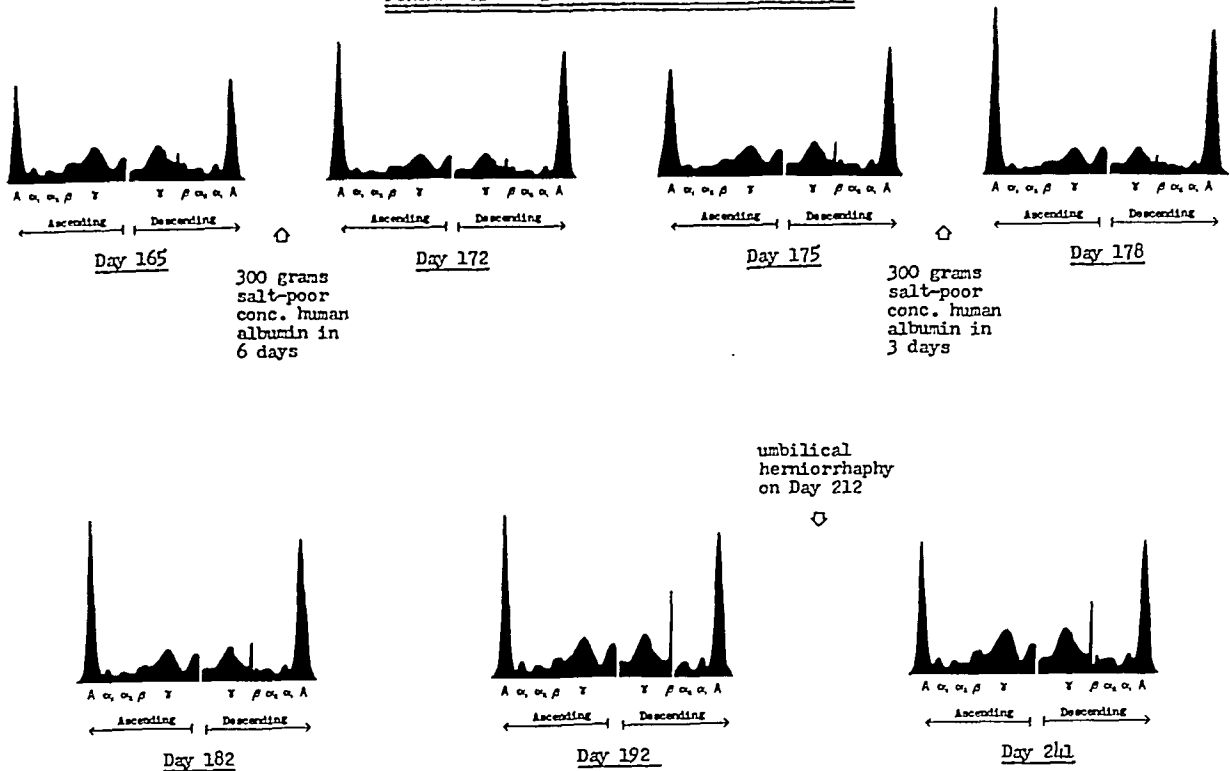
analysis of R. M. S. than in any of the other patients in the group, presumably because of the previous therapy. After the administration of 3000 cc. of plasma, the total proteins rose, with increases in both the relative and absolute amounts

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

P.P. - 51 year old male. Chronic alcoholic. Marked ascites with repeated paracenteses before admission. Plasma and blood transfusions. Regular diet until Day 108; full dietary regime from Day 107 on.

FIG. 5a

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT



P.F. (continued) - Infusions of salt-poor concentrated human albumin during dietary regime. Complete clinical recovery with disappearance of ascites. After discharge patient had partially strangulated umbilical hernia repaired on Day 212 with uneventful recovery.

Fig. 5b

of albumin and gamma-globulin, and in the absolute amounts of alpha-2- and beta-globulins (Figure 4 and Table IV).

had anasarca and massive ascites, but unlike the other subjects in the group, the serum bilirubin was not elevated.

P. F.—51 year old male. Ascites had been present for nine months and was increasing despite repeated paracenteses. On admission, the patient

From Day 0 through Day 86 the patient received a "regular" diet (C 293, P 70, F 62, Cal. 2010) which was, nevertheless, more than he had

TABLE IV

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

Sera of R.M.S.

	Electrophoretic data										Howe sodium sulfate										
	per cent					grams per cent					A/G	grams per cent			bilirub		ceph	thym	alk phos	BSP %	
	Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ		A/G	Alb	Glob	Tot	dir					tot
Day 0	46.8	6.7	9.1	17.4	20.0	2.48	0.36	0.48	0.93	1.06	0.88	1.35	3.05	2.26	5.31	2.1	2.8	2+	5.2	9.0	--
p																					
Day 14	48.0	5.0	9.0	15.9	22.1	3.32	0.35	0.62	1.10	1.53	0.92	1.37	4.00	2.92	6.92	2.1	2.3	2+	13.2	8.2	70

P = 3000 cc. of plasma in 9 days

TABLE V

PORTAL CIRRHOSIS UNDER MEDICAL MANAGEMENT

Sera of P.F.

	Electrophoretic data											Howe sodium sulfate									
	per cent					grams per cent					A/G	grams per cent									
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ		A/G	A/G	Alb	Glob	Tot					
																	bilirub dir	tot	ceph	thym	alk phos
Day 0	36.2	7.1	7.5	17.4	31.8	1.88	0.37	0.39	0.91	1.66	0.57	0.94	2.53	2.68	5.21	0.2	0.3	2+	6.8	3.9*	
Day 20	32.7	9.5	12.3	15.3	30.2	1.76	0.51	0.66	0.82	1.63	0.49	0.77	2.34	3.04	5.38	---	---	3+	---	4.7	
Day 35	37.2	8.3	10.1	13.8	30.6	2.09	0.47	0.57	0.78	1.72	0.59	0.88	2.63	3.00	5.63	0.4	0.7	2+	4.8	7.8	
Day 50	34.9	8.2	10.1	15.3	31.5	2.03	0.48	0.59	0.89	1.83	0.54	1.16	3.12	2.70	5.82	0.5	0.9	3+	6.2	3.7	
Day 75 _p	38.4	8.0	9.7	12.1	31.8	2.33	0.49	0.59	0.74	1.93	0.62	0.69	2.90	4.18	6.08	---	---	1+	5.2	---	
Day 105 _b	43.6	5.7	6.3	14.9	29.5	3.17	0.42	0.46	1.09	2.15	0.77	1.02	3.69	3.60	7.29	0.4	0.7	--	---	4.2	
Day 136 _a	42.0	6.3	7.5	10.2	34.0	2.54	0.38	0.45	0.62	2.06	0.72	1.27	3.38	2.67	6.05	0.4	1.1	2+	5.2	4.4	
Day 165 _a	39.2	6.7	7.3	12.5	34.3	2.77	0.47	0.52	0.89	2.42	0.65	0.89	3.32	3.75	7.07	0.1	0.8	3+	4.7	4.0	
Day 172 _a	54.4	4.9	5.5	10.1	25.1	4.00	0.36	0.40	0.74	1.84	1.19	1.57	4.48	2.85	7.34	2.5	1.1	1+	4.8	---	
Day 175 _a	49.7	5.4	6.0	10.9	28.0	3.72	0.40	0.45	0.82	2.10	0.99	1.11	3.94	3.55	7.49	0.4	1.3	3+	4.8	---	
Day 178 _a	60.3	4.7	5.8	7.5	21.7	4.43	0.35	0.43	0.55	1.60	1.52	2.00	4.91	2.45	7.36	0.4	1.8	3+	3.9	---	
Day 182	53.0	5.2	5.6	9.0	27.2	3.76	0.37	0.40	0.64	1.93	1.13	1.52	4.28	2.82	7.10	---	---	3+	5.8	---	
Day 192	45.6	5.9	6.6	9.7	32.2	3.66	0.47	0.53	0.78	2.59	0.84	1.02	4.06	3.97	8.03	0.5	1.5	3+	9.0	---	
Day 241	40.8	5.9	6.7	12.7	33.9	3.43	0.49	0.56	1.07	2.85	0.69	1.05	4.30	4.10	8.40	---	---	3+	7.5	---	

p = 8000 cc. of plasma in 12 days

b = 3600 cc. of whole blood in 10 days

a = 300 grams of salt-poor concentrated human albumin

* starred values not simultaneous with other data

been eating before hospitalization. Partial clinical improvement occurred, with slight diminution of ascites. There was almost 1 gram per cent rise in the total proteins, but relatively little change toward normal in the proportions of the fractions as determined by five electrophoretic analyses (Figures 5a and 5b, and Table V). Experiments on electrolyte and water metabolism (to be reported separately) were performed during this period with no significant effect on the electrophoretic pattern.

In the 12-day period of *Day 87* through *Day 98* while on the "regular" diet, the patient received 8000 cc. of plasma. The electrophoretic pattern of *Day 105* revealed for the first time a considerable change toward normal.

From *Day 108* on the patient was on the full dietary regime (C 437, P 155, F 123, Cal. 3475 supplemented by 5 grams choline chloride). In the ten-day period of *Day 126* through *Day 135* he received 3400 cc. of whole blood. There was no further change of the electrophoretic pattern toward normal (*Day 136* and *Day 165*), but on the contrary, some retrogression.

The intravenous administration of 300 grams of salt-poor concentrated human albumin in six days (*Day 166* through *Day 171*) was followed by a striking elevation of the serum albumin in the electrophoretic pattern of *Day 172*, while both the relative and absolute values of all globulin fractions were diminished, presumably due to dilution. Three days later (*Day 175*), the electrophoretic pattern revealed some decline of the serum albumin and rise of the gamma-globulin and other globulin fractions.

After a second course of 300 grams of salt-poor concentrated human albumin, administered in three days (*Day 175* through *Day 177*), a striking elevation of the serum albumin and a diminution of all the globulin fractions again occurred. Once more this was followed by some decline of the serum albumin and a rise of the gamma-globulin and other globulin fractions, this trend being clearly evident in the patterns of *Days 182*, *192*, and *241*, respectively five, 15, and 64 days after the last infusion of albumin. The ascites disappeared and the patient was discharged on *Day 192* completely free of clinical symptoms, and he resumed

his former occupation. A partially strangulated umbilical hernia was repaired on *Day 212* with uneventful recovery. In spite of this episode the final electrophoretic analysis of *Day 241* indicated a higher absolute value for serum albumin than at any time prior to institution of the human albumin therapy, and a higher total protein than at any time. Six months after the last albumin infusion the patient remained well, having gained weight with no recurrence of edema or ascites.

Group II. The five cases of portal cirrhosis with long-term clinical recovery under medical management are summarized briefly in Figure 6. The patients had been severely ill with hepatic insufficiency before treatment; four had ascites requiring paracenteses and one had pronounced jaundice; all had hypoalbuminemia, the mean of the albumin values of the five patients being 2.58 grams per cent by Howe sodium sulfate fractionation. As illustrated in Figure 6 and Table VI, the electrophoretic patterns after sustained re-

covery appear very nearly normal with but minimal deviations from the normal range.

DISCUSSION

The five patients in Group I initially had marked diminutions of the albumin fractions and elevations of the gamma-globulin fractions, with somewhat less elevated beta-globulins. During the course of medical management the serum proteins underwent progressive evolution in the direction of normal, coinciding with clinical improvement. The progress of the three patients in the group treated with diet alone indicates that this is not a perfectly strict relationship. J. B., who improved only partially (the ascites disappearing, but the jaundice persisting), had fully as pronounced a change of the electrophoretic pattern toward normal as did J. K. with complete clinical recovery. Moreover, M. S., whose clinical improvement was more marked than J. B.'s, had less evolution toward a normal serum protein composition, the

PORTAL CIRRHOSIS WITH LONG-TERM CLINICAL RECOVERY UNDER MEDICAL MANAGEMENT

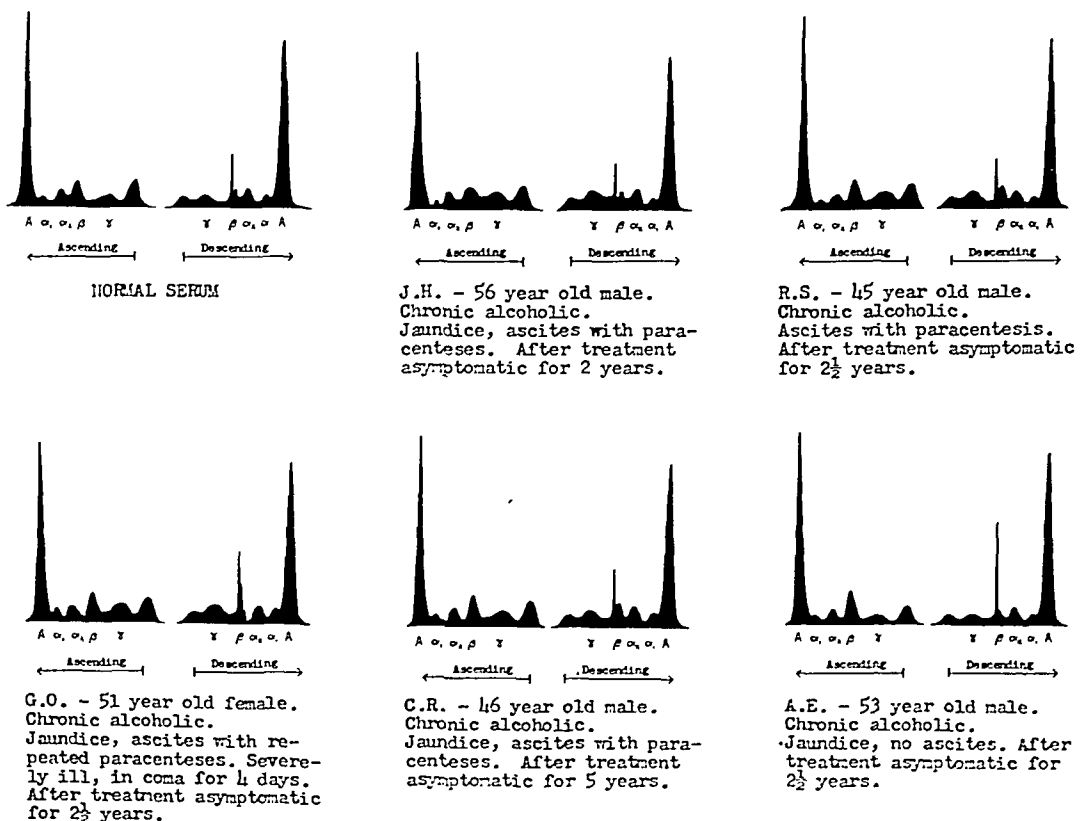


FIG. 6

TABLE VI

PORTAL CIRRHOSIS WITH LONG-TERM CLINICAL RECOVERY UNDER MEDICAL MANAGEMENT

	Electrophoretic data											Howe sodium sulfate									
	per cent					grams per cent					A/G	grams per cent									
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ		A/G	A/G	Alb	Glob	Tot	bilirub dir	tot	ceph	thym	alk phos
J.H.	56.4	3.5	9.6	15.8	14.7	3.87	0.24	0.66	1.08	1.01	1.29	1.45	4.06	2.80	6.86	0.5	0.7	2+	5.3	4.7	6
R.S.	58.0	4.3	8.4	14.5	14.8	3.90	0.29	0.56	0.98	1.00	1.38	1.85	4.37	2.36	6.73	0.3	0.6	3+	9.8	2.8	13
G.O.	55.9	5.4	8.4	14.9	15.4	4.05	0.39	0.61	1.08	1.11	1.27	1.76	4.62	2.62	7.24	0.2	0.6	1+	6.3	5.9	34
C.R.	55.2	5.4	10.2	16.2	13.0	3.84	0.38	0.71	1.12	0.90	1.23	1.74	4.41	2.54	6.95	0.2	0.7	neg.	7.9	4.2*	--
A.E.	62.6	4.0	8.2	16.8	8.4	4.13	0.26	0.54	1.11	0.55	1.68	2.62	4.77	1.82	6.59	0.1*	0.6*	neg.	1.3	2.6	3
AVERAGE	57.6	4.5	9.0	15.6	13.3	3.96	0.31	0.62	1.07	0.91	1.37	1.88	4.44	2.43	6.87	* starred values not simultaneous with other data					
Standard Deviation	0.8	0.8	0.8	1.1	2.6	1.10	0.06	0.06	0.05	0.19	0.16	0.39	0.24	0.34	0.22						

NORMAL SERA

(10 volunteer subjects with normal liver function tests)

AVERAGE	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93	1.52	2.31	4.89	2.12	7.01						
Standard Deviation	2.8	0.8	1.5	1.2	1.7	0.29	0.06	0.10	0.08	0.13	0.18	0.34	0.30	0.24	0.30						

gamma-globulin fraction remaining markedly elevated.

The two cases in Group I considered to have achieved complete clinical recovery, J. K. and P. F., continued to have appreciable abnormalities in the serum protein composition. That the serum protein composition may eventually become normal or nearly so in cases with long-term clinical recovery under medical management is indicated by the five patients in Group II (Figure 6). At the time of admission with hepatic insufficiency these patients all had marked alterations of the serum proteins by the Howe fractionation, which is less sensitive than the electrophoretic technique.

The evaluation of changes in the serum proteins in edematous patients is hazardous unless account is taken of the possibility of shifts in body fluids. Thus an increase in protein concentration may reflect either an increase in the circulating protein or a decrease in the volume of the plasma.

During the observation of P. F., 16 estimations of the plasma volume were made with Evans blue,

to be reported separately in connection with studies of electrolyte metabolism. The findings did not indicate that changes of plasma volume would account for the progressive rise of total proteins on dietary therapy or infusions.

In J. K., J. B., and M. S., the three cases treated by diet without infusions, the electrophoretic data alone exclude the possibility that the rise in albumin and total proteins could be solely due to hemoconcentration. These three cases exhibited rise of albumin and decline of gamma-globulin, a circumstance not explicable in terms of any possible fluid shift, and indicating changes in protein metabolism.

In the infusion experiments it appears probable that transitory changes in plasma volume did play a role. The infusion of plasma was followed by increases in the albumin and the beta- and gamma-globulin fractions as well as the total proteins. The immediate effects of intravenous administration of salt-poor concentrated human albumin on the serum protein composition were

identical with those observed by Thorn, Armstrong, and Davenport (6), *i.e.*, marked elevation of the albumin and depression of the globulin fractions; presumably due to dilution. The changes beginning shortly thereafter consist of some decline of the albumin and rise of the gamma-globulin and other globulin fractions. These ensuing alterations have apparently not been described by previous investigators of human albumin infusion therapy. The phenomenon was observed in the two administrations of human albumin to P. F. and also in a patient with cirrhosis of uncertain etiology not included in the present study. The significance of these observations remains to be determined. It is possible that as the administered albumin left the circulation the plasma volume decreased with resultant concentration of the globulin components.

Comparison of the alterations of the serum proteins with the various tests of hepatic function performed simultaneously fails to reveal any clearly evident direct correlation.

SUMMARY

1. The effects of medical management on the serum proteins in ten proven cases of portal cirrhosis have been investigated by the electrophoretic method.

2. Serial electrophoretic analyses of the sera of patients with ascites revealed progressive change in the direction of normal, coinciding with clinical improvement during the administration of a high protein, high carbohydrate, high caloric diet for periods ranging up to ten months.

3. Infusions of plasma or salt-poor concentrated human albumin were followed by pronounced and immediate elevations of the serum albumin values.

4. Shortly after the administration of human albumin, some decline of the elevated albumin value was observed, with rise of the gamma-globulin and other globulin fractions.

5. In five patients who had clinically recovered from severe portal cirrhosis and had been asymptomatic for more than two years the electrophoretic patterns were very close to normal.

6. The alterations in the serum protein pattern in portal cirrhosis are not irreversible; with prolonged medical management and clinical recovery, the proteins may revert practically to normal.

The authors wish to express their gratitude to Doctor E. S. Guzman Barron for making available the electrophoretic apparatus used in this study.

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PRODUCTION AND AVAILABILITY OF RADIOISOTOPES

By PAUL C. AEBERSOLD

(From the Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tenn.)

Radioisotopes as tracer atoms and as uniquely applicable sources of radiation have become important working tools of clinical investigation. Indeed, they have already been established as permanent tools, indispensable to many investigations. They provide a new and dynamic power of perception—an ultra-sensitive way of following certain atoms. Their application has led to the development of new and unique techniques for studying the metabolism of a wide variety of elements and biochemical compounds, diagnosing and treating disease and studying the behavior and role of pharmaceuticals and other medicinal agents. However, the greatest value of isotopes is expected to be derived from their contribution to the over-all advance of medical knowledge.

PRODUCTION

The uranium chain-reacting pile has two unique characteristics, the production of a fission chain reaction and the production of vast quantities of neutrons. Both of these features make the reactor a useful and extremely productive unit for making radioisotopes; the first for yielding the so-called "fission products," and the second for producing artificially induced radioactivity. It is the second feature, however, which permits the production of the greater variety of radioelements and the production of isotopes of elements generally found in biological material.

Even before the last war and before the advent of atomic energy reactors, radioisotopes were, of course, producible with high energy "atom smashing" devices, such as the cyclotron. Indeed, investigators using cyclotron-produced isotopes had found in them the most sensitive tool ever placed at their disposal. They also found the radioisotope to be a tool which will permit tracing a specific batch of atoms through a complicated physical, chemical or biological system even though the radioactive atoms may become mixed with many other stable atoms of the same element.

There was one drawback, however, to their widespread utilization at that time. Cyclotron production of radioisotopes was and still is both

limited and costly. Thus at the start of the war, science had in radioisotopes a research tool guaranteeing high sensitivity and high specificity—a tool in great demand but in small availability.

During the wartime development of the atomic energy project it became evident to scientists who worked with the chain-reacting uranium pile that it would be an excellent unit for producing huge quantities of radioactivity or radioisotopes—for making these valuable tools available on a very wide scale. Out of this realization grew the program for the distribution of pile-produced radioisotopes which was initiated by the Manhattan Project and further developed by the Atomic Energy Commission.

As indicated earlier, radioactive isotopes are created in chain-reacting piles by two processes (1) fission of uranium 235 or other fissionable nuclei and (2) neutron absorption by non-fissionable nuclei placed in the pile for the purpose.

Fission Process

The fission process results generally in two radioactive fragments, one to three neutrons and several gamma rays as indicated in Figure 1. The total mass of the fragments plus the mass of the one to three neutrons which are emitted during the fission event is equal to the mass of the U 235 nucleus (except for the loss of about one-tenth of 1 per cent of the mass which is converted into energy and which causes the tremendous release of energy during fission). The fission fragments are very unstable nuclear species ranging from element number 30, zinc, to element number 64, gadolinium. Each of these species approaches a stable nuclear state by a series or chain of beta ray emissions. The intermediate decay products formed during the beta chain decay are the fission products.

These fission products, because they differ chemically from the original uranium and other possible transuranic contaminants, may be isolated in groups or as isotopes of a single element. The only radioisotope of biological significance produced in this way, however, is radioiodine, I^{131} .

URANIUM FISSION AND BETA CHAIN DECAY

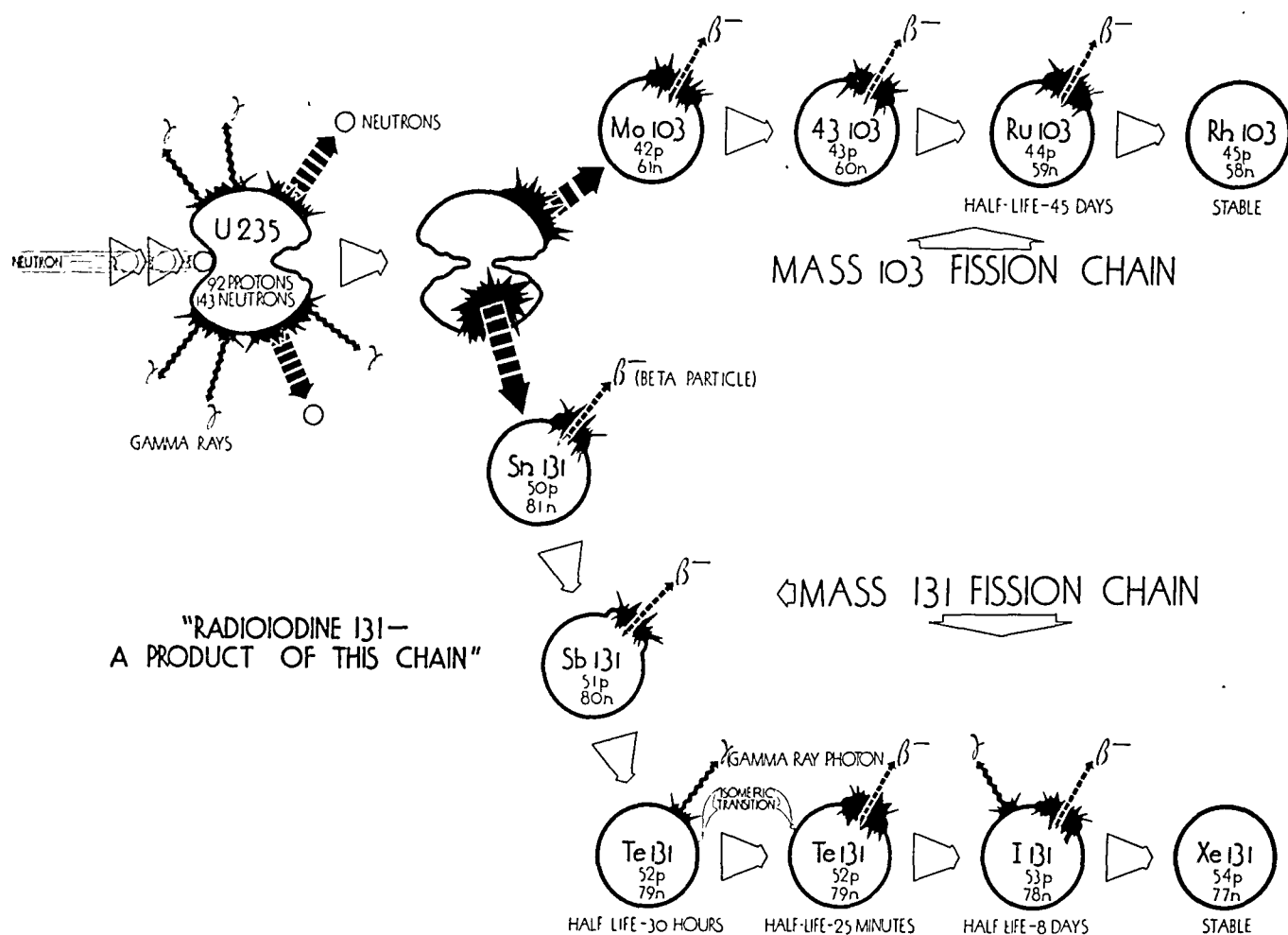


FIG. 1. URANIUM FISSION CHAIN REACTION

When Uranium 235 fissions it splits into two fragments which may range in weight from 65 to 157 or from element number 30, zinc, to element number 64, gadolinium. On the basis of probability, however, a greater percentage of the fragments range from element number 38 to 45, and from number 54 to 61. Each of the radioactive fragments approaches a stable nuclear state by a series or chain of beta ray emissions. The intermediates formed during this decay are the source of the fission products, such as radioiodine 131. Although the above illustration shows only one of many ways in which a $\text{U } 235$ atom may fission, it was chosen as a single example in which a conservation of mass and number of nuclear particles can be shown. It is also an excellent example of how a biologically useful radioisotope, iodine 131, may be produced through uranium fission.

The production of radioiodine as a fission product is shown in Figure 1.

Neutron Activation

The pile production process which yields radioisotopes of a much wider variety of elements, in fact, radioisotopes of most of the elements from hydrogen to plutonium, is neutron absorption. Generally, however, the neutron absorption process is subdivided into two categories: (1) simple neutron capture in which only a gamma ray photon is emitted and the product is hence an isotope of the target element and (2) transmutation in which

a proton or alpha particle is emitted and the product is hence an isotope of a different element than the target.

The neutron absorption process of making radioisotopes in the pile is extremely prolific because of the pile's capacity to produce tremendous quantities of neutrons. Indeed, neutrons can be produced greatly in excess of those needed to sustain the chain reaction, and these excess neutrons are available for other uses, such as the bombardment of stable materials to form radioactive materials. In the pile at the Oak Ridge National Laboratory where most of the radioisotopes distributed by

the Atomic Energy Commission are prepared, the "flux" or density of the "atmosphere" of neutrons inside the pile is approximately 10^{12} (a million million) neutrons flowing through a square centimeter each second. This value seems even more impressive when one considers that this density exists in a volume occupied by many tons of graphite and uranium.

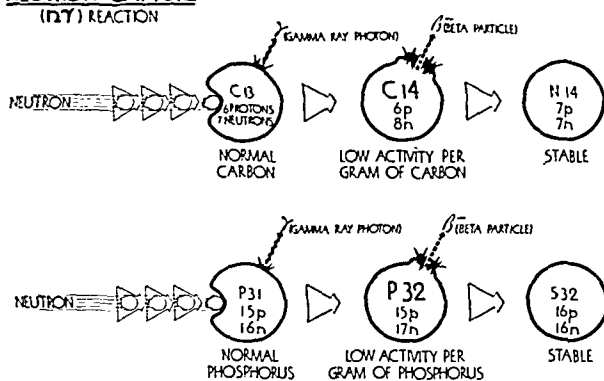
For the details of production of radioisotopes by neutron absorption, attention is called to Figure 2. As indicated in the illustration, ordinary elements like carbon and phosphorus may be changed

into their radioactive counterparts by the absorption of a neutron and the emission of a photon of gamma radiation. This reaction, usually referred to as an (n, gamma) reaction, is by far the most prevalent production reaction used in radioisotope preparation. It is not, however, the most practical in all cases. For instance, in the production of radioactive carbon 14 by the (n, gamma) reaction, a neutron must be absorbed by the stable isotope, carbon 13. Although carbon 13 is found in all naturally occurring carbon, it has a normal abundance of only 1.1 per cent. To obtain an efficient yield of carbon 14 by this method would therefore necessitate bombarding a carbon target whose carbon 13 content had been concentrated many times above this abundance. This procedure would, however, prove time-consuming and expensive. Furthermore, it is even less feasible in light of the fact that the carbon 13 isotope has an extremely low probability for capture of "slow" or thermal energy neutrons (low neutron capture cross section). With radioactive phosphorus 32, however, the situation is quite different, in fact the (n, gamma) reaction is very practical for its production. Because naturally occurring phosphorus has only one stable isotope (phosphorus 31) and because this isotope has a high probability for capturing thermal energy neutrons, a good yield of radioactive phosphorus results from the (n, gamma) reaction.

The radioisotope formed by an (n, gamma) reaction is merely the radioactive counterpart of the stable element used as the target material. The radioisotope in this case, therefore, will always be diluted with the stable atoms of the target element which have not been made radioactive during the pile irradiation. Because both the radioactive and stable atoms are of the same element, no chemical separation can be effected. The resultant activity per gram of element is called the "specific activity." It is dependent on the chance for capture of a neutron (capture cross section) by the target nucleus, the neutron "flux" of the production pile and the duration of the irradiation. The specific activity of a radioisotope may be extremely important for its use as a tracer because it determines to what extent the radioisotope may be diluted and still afford reliable detection and measurement.

PILE PRODUCTION OF RADIOISOTOPES

NEUTRON CAPTURE (n, γ) REACTION



TRANSMUTATION (n, p) REACTION

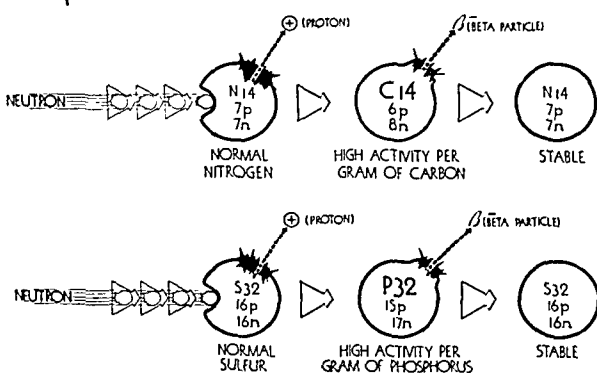


FIG. 2. PILE PRODUCTION OF RADIOISOTOPES

There are two ways of producing radioisotopes by neutron absorption in a nuclear reactor. The first, neutron capture, is effected by the capture of a neutron and the emission of a gamma ray photon yielding a radioisotope of the same element as the target material. The second, transmutation, is effected by the capture of a neutron and the emission of a proton or alpha particle yielding a radioisotope non-isotopic with the target element. Radiophosphorus 32 and radiocarbon 14 may be produced by either or both of these reactions.

On the other hand, a radioisotope produced by a transmutation reaction is an isotope of a different element than the target element (non-isotopic with the target element). It may, therefore, be chemically separated from the residual stable atoms in the target after pile irradiation. A radioisotope produced in this way and then chemically separated from the irradiated material, *i.e.*, from the stable isotopes of the same element, is called "carrier free." In practice some "carrier" or stable element is usually added to facilitate radiochemical handling. Nevertheless, radioisotopes produced by transmutation have a much higher specific activity than those produced by an (n, gamma) reaction. For this reason, transmutation is a particularly useful production reaction. The chemically extracted transmuted radioisotope will be diluted only by stable element present as impurity in the compound irradiated, or by stable element added as carrier to help extract the radioisotope. It can thus undergo great dilution in a tracer experiment before becoming undetectable.

As also indicated in Figure 2, radiocarbon 14 and radiophosphorus 32 may be produced by a transmutation reaction. In both cases a neutron is absorbed and a proton is emitted during the reaction. In the case of carbon 14 production, the target material is a nitrogen compound (calcium nitrate) while for phosphorus 32 the target material is elemental sulfur. Both of these target nuclei have a high natural abundance in the element and both target materials are readily available and may be obtained quite pure chemically.

There is still another type of transmutation reaction used in the pile production of radioisotopes—the reaction in which a neutron is absorbed and an alpha particle is emitted. This reaction, however, is feasible for the production of only two isotopes, radiohydrogen (H 3 or tritium) from lithium 6 and radioargon (A 37) from calcium 40.

The mechanical operations associated with pile production of radioisotopes is quite simple. Reference is made to Figure 3 showing a schematic diagram of the pile at the Oak Ridge National Laboratory which is used to make most of the radioisotopes now being distributed by the Commission. In the case of the fission products, the radioisotopes are chemically extracted from the active uranium slugs which have previously been

discharged into a channel of water at the rear of the pile.

If the radioisotope has resulted from an (n, gamma) reaction, it is usually shipped as an irradiated unit. This means that the material in the aluminum test tube, consisting of stable atoms of the target material together with their counterparts which have been made radioactive, is merely removed and packaged prior to shipping. If, on the other hand, the radioisotope has been produced by a transmutation reaction, it may be chemically separated from the target material prior to packaging and shipping.

Production Status

When the radioisotope distribution program was initiated, production scheduling was based primarily on the expected demand of medical investigators. To a great extent the same basis is used in scheduling current production. At least two reasons account for this situation. In the first place a majority of potential radioisotope users were and still are associated with some phase of medical or biological research. In the two and a half year period since the inauguration of the distribution program, nearly 75 per cent of the radioisotope shipments have been used for studies in medical therapy, animal physiology or bacteriology.

The second reason for special effort in preparing the radioisotopes needed by medical research is associated with the characteristics of the isotopes generally used in these investigations. Many of the biologically useful radioisotopes are short lived materials, that is, over a comparatively short period of time most of the radioactivity of the isotope will have disappeared through spontaneous disintegration. Indeed, if the radioisotope is to be used in human studies, it is desirable that it have a short life of radioactivity in order to prevent extended radiation exposure of the patient. It is difficult, however, to supply materials of short half-life because they cannot be stockpiled for an appreciable length of time. They must be produced currently to meet current and fluctuating demands. Of the total number of radioisotope shipments made thus far over 65 per cent have been shipments of phosphorus 32 or iodine 131, having respective half-lives of 14.3 days and 8.0 days.

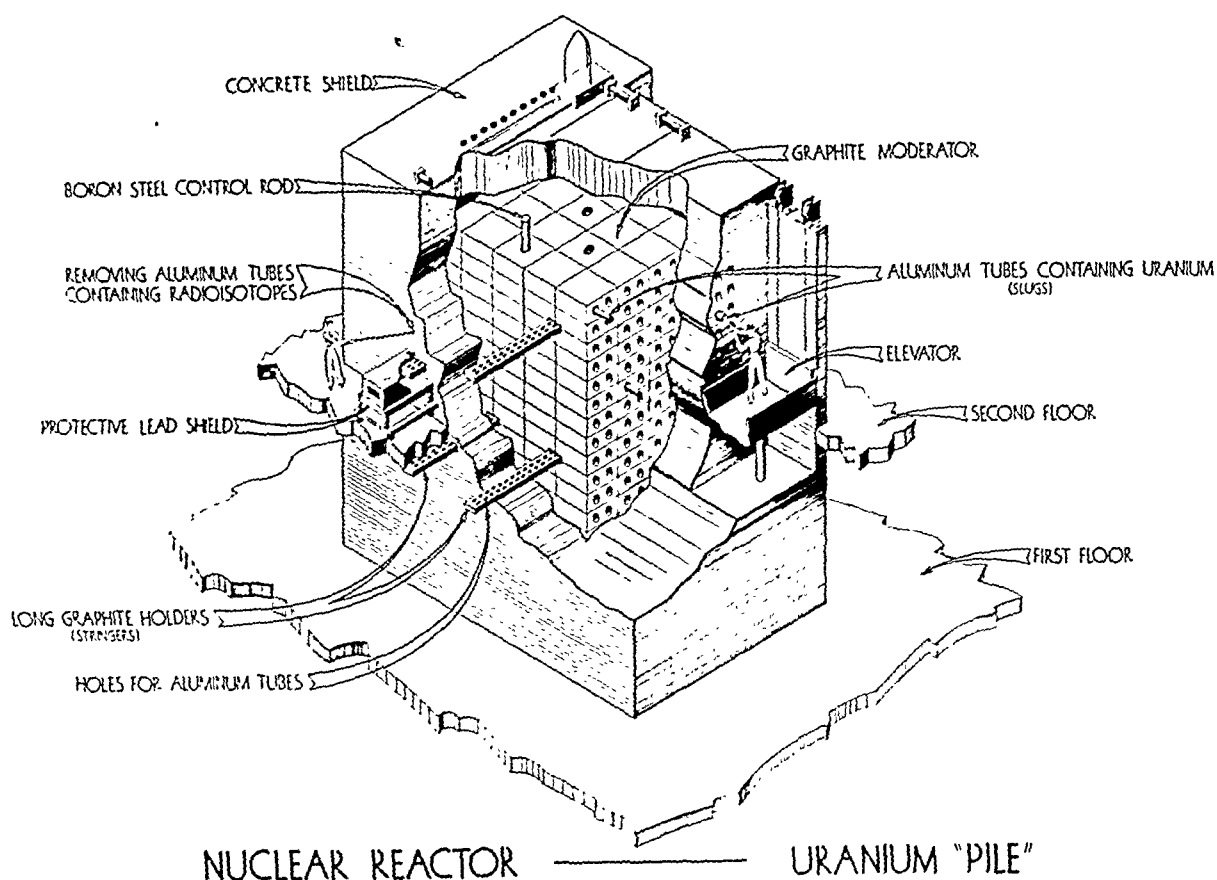


FIG. 3. NUCLEAR REACTOR—URANIUM PILE

The functional parts of the nuclear reactor are the fissionable material (uranium), the moderator material (graphite) and the control rods (boron steel). The uranium slugs are inserted into the pile from the right face (where the elevator is located). They are discharged at the opposite side of the pile into a channel located between the graphite and concrete. The slugs drop into water and are later chemically treated for removal of the fission products. The radioisotopes formed by the neutron bombardment of stable isotopes are irradiated in graphite holders or stringers as indicated on the left. The control rods are lowered into the pile to retard or stop the chain reaction. The thick concrete wall surrounding the reactive portion of the pile serves as protection for operating personnel.

Most of these shipments have been for medical and clinical investigations.

Today, pile production of radioisotopes has been stepped up to a point where these valuable research tools are available to all qualified investigators. In fact, the situation existent before the advent of pile production, when there was not an adequate supply of radioisotopes to meet the demands of qualified investigators, has been reversed. The present limitation to wider use of radiomaterials is the lack of personnel having the special training and facilities to use them safely. And yet, the number of qualified investigators has been multiplied several times above the prewar level.

AVAILABILITY

Radioisotopes are potentially hazardous to health and for this reason special facilities and techniques are required in handling and using them. The Atomic Energy Commission's primary concern in making radioisotopes available is that they will be used safely. To insure this provision, the Commission has set up certain criteria for the allocation of radioactive isotopes. The Commission has also procured the services of an Advisory Committee on Isotope Distribution to assist in the allocation of these valuable and yet potentially hazardous materials.

The Subcommittee on Human Applications, a subcommittee of the main Advisory Committee, reviews all radioisotope requests proposing use of radiomaterials in humans. This subcommittee has outlined the following general aims governing the distribution of radioactive materials for medical purposes: (1) to make radioisotopes available for approved clinical investigations and for diagnostic and therapeutic purposes where they are needed and can be handled properly and safely; (2) to protect, as far as possible, patients from indiscriminate use of radioisotopes; and (3) to insure that adequate facilities are available for those planning to use the material.

In carrying out these very worthwhile aims the Commission is faced with two opposing purposes: first, to make these materials widely available and of maximum usefulness to medical research and second, to limit distribution sufficiently to guarantee their safe utilization. To achieve the desired balance between these two somewhat contradictory purposes, the Subcommittee on Human Applications has formulated criteria for allocation.

Criteria of Allocation

These criteria have proved satisfactory for the administration of the distribution program. Their merit is clearly evident from enumeration of the requirements: (1) physicians using radioactive material must be associated with a medical research institution, hospital, clinic or other organization having adequate facilities for handling the material; (2) such facilities must provide clinical care of the patient and adequate means for assaying, safely handling and disposing of the radioactive material; (3) the organization requesting the radioisotopes must be in good standing with the local medical society; (4) it is also strongly recommended that the requesting institution appoint a clinical "Isotope Committee" to evaluate all proposals for clinical use of the material within the institution; (5) the scientifically trained individual who will use or directly supervise the use of radioactive material must be an accredited physician in good standing with the local medical society; (6) also, the physician must have had previous clinical experience with radiation or radioactive materials or be directly collaborating with an individual possessing such training and experience.

In connection with the above recommendation the Subcommittee on Human Applications has recently adopted the policy of not approving a radioisotope request unless it bears a written statement of approval from the local clinical isotope committee. It is suggested that this local committee includes: (a) a physician trained in internal medicine; (b) a physician trained in hematology; (c) a person experienced in assay of radiomaterials and protection of personnel against ionizing radiations; and, whenever possible, (d) a qualified physicist, and (e) a therapeutic radiologist. If a physicist and radiologist are not staff members of the requesting institution, they should be available in a consulting capacity.

Information indicating that the requesting institution meets these qualifications together with more detailed information concerning the proposed use of the material and proposed health safety measures is forwarded by the applicant in his formal request. Following review and approval of the request by the Subcommittee on Human Applications, the applicant is authorized to procure the material. Subsequent arrangements concerning purchasing the material and scheduling of shipment are made between the applicant and the supplier.

If the radioactive material is not intended for use in humans, the request is not reviewed by the Subcommittee on Human Applications. For animal studies, as well as for all other types of radioisotope investigations, prior reference of all requests to a local isotope committee is strongly recommended. In institutions where clinical and non-clinical investigations are carried on by different groups, a separate isotope committee may be established for the control and coordination of the use of isotopes in the non-clinical studies. Review by a local committee assures the Commission that the proposed program is considered to be both feasible and safe from the standpoint of the institution.

Basic and Special Products

When the Atomic Energy Commission inaugurated its distribution program, it focussed its attention primarily on producing a wide variety of isotopes rather than on producing a wide variety of compounds of a small number of isotopes. Indeed, early production was aimed at making

the largest number of isotopes available in the simplest and most generally adaptable forms. By thus placing emphasis on producing simple basic forms for each isotope it has been possible to make these "basic products" available cheaply and on a large scale.

In many instances, the radioisotope users can conveniently use the materials in the simple basic forms as shipped. Radioiodine (I 131) and radio-phosphorus (P 32), for instance, are often clinically administered as solutions of sodium iodine and disodium hydrogen phosphate which are the chemical forms in which the materials are shipped from the production site. In other cases, however, users may require a modified form or even a specially synthesized compound containing the isotope.

These specially synthesized compounds containing radioisotopes, "labeled" compounds, are extremely important to clinical and medical investigations because they permit tracer studies to be made of the molecules and parts of molecules which enter into biochemical and physiological processes. The element carbon, for instance, is one of the body's major constituents, although, of course, not in its elemental form. Indeed, it is always found in some complex molecule such as an amino acid or sugar. Therefore, in using radioactive carbon 14 to study a certain body compound it is necessary first to incorporate the radioisotope in the specific compound to be studied. These labeled or tagged compounds can be classed as "special products." They may vary from simply the modification of the material shipped, say from the carbonate into sulfate or sulfide, to the complicated synthesis of complex organic molecules.

The Commission has not undertaken an extensive program within its own facilities to prepare and make available these special products. It would not only be difficult for the Commission to maintain facilities and personnel to meet all the special needs of off-Commission users, but this is an area of endeavor which is free of security considerations and one which logically should be handled by private enterprise. Limited quantities of labeled compounds which are obtained as by-products of Commission research activities are made available to off-Commission laboratories when prepared in excess of Commission needs.

The Commission has, however, encouraged commercial firms to engage in the preparation of special products. At the present time three organizations outside Commission laboratories are preparing and distributing isotope-labeled compounds and several other organizations are considering arrangements for initiating similar programs. There are over 50 such compounds now available and 35 additional compounds are scheduled to become available within the next six months. Although most of these compounds are labeled with radiocarbon (C 14), some compounds have been labeled with radioisotopes of sulfur, iodine, gold, zinc and cobalt.

Even though allocation of these materials is necessarily controlled by the Commission, all other arrangements relative to their procurement are made by the supplier and the radioisotope user. Also in its effort to encourage preparation and sale of isotope-labeled compounds by private enterprise, the Commission has adopted the policy of discontinuing distribution of any compound which becomes available from an outside source.

Besides its efforts to encourage wider use of radioisotopes through cooperating with private enterprise in programs such as mentioned above and through its support of training and educational programs, the Commission has also stepped up and amplified its own program of producing basic radioisotope products. New facilities for producing and processing these products are being designed and constructed. The Commission has made an effort to encourage the growth of medical and biological research and evidence of this was noted last April when it was announced that radioactive phosphorus (P 32), radioactive iodine (I 131), and radioactive sodium (Na 24) would be made available free of production costs for use in therapy, diagnosis, and research in cancer and allied diseases. More recently a further announcement was made stating that this program would not only be continued but expanded to include all isotopes normally available under its isotope distribution program providing the material is used in cancer research. More specifically the supplementary extension of the program provides for allocation of materials free of production cost for use in (1) cancer investigations involving animal

subjects, (2) research programs studying basic cellular metabolism of cancerous cells, and (3) experimental programs designed to evaluate the uses of radioactive materials in the therapy and diagnosis of cancer.

CONCLUSION

In pointing out the present widespread availability of radioisotopes as a research tool, it must also be noted that this is based on much more than merely the increased availability of isotopes per se. Not only are radioisotopes available in amounts many times the prewar quantities, but they are also readily available as a much greater variety of radioelements. Nearly all the biologically useful radioisotopes, including such important ones as radiohydrogen (tritium, H 3), radiocarbon (C 14), radiophosphorus (P 32), radiosulfur (S 35), radiocalcium (Ca 45), radioiodine (I 131), etc., are produced routinely and are available continuously.

Further, a stock of isotope-labeled compounds is available for the first time. Many complex organic molecules have been labeled or tagged with iso-

topes and many more will become available in the near future. Heretofore, tracer studies with isotopes were usually limited to following only the radioelement or its simple compounds. The availability of a wide variety of labeled organic and biochemical compounds will certainly promote much more extensive and diversified utilization of isotopes in biology and medicine.

Increased availability of materials and instruments has led recently to a much greater number of hospitals, clinics and other institutions using radioactive materials in routine diagnosis and therapy procedures and in research investigations. Before the war, for instance, little more than a dozen institutions were routinely using radiophosphorus (P 32) and radioiodine (I 131) in diagnosis and therapy. Today 80 different institutions are using either or both of these isotopes in this type of clinical work.

It seems quite certain that with the background at hand and the impetus now gained, future research with isotopes, especially isotope-labeled compounds, will lead to valuable new routine uses of radioisotopes in diagnosis and therapy.

THE USE OF STABLE ISOTOPES IN BIOLOGICAL AND MEDICAL RESEARCH

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During the past 15 years there has been a rapid and extensive development of the use of isotopes in biological and medical investigation. In 1923, Hevesy's pioneering studies, in which thorium B was used to investigate the absorption and localization of lead by plants, revealed some of the possibilities of the use of tracer technique for biochemical research. The exploitation of these possibilities marked time, however, until isotopes of elements more widely associated with biochemical processes were made available. The discovery of deuterium by Urey in 1931 and the development of methods for production of heavy water were the first steps toward providing stable isotopes for tracer studies in biological systems.

There soon followed the preparation of the heavy isotopes of nitrogen, carbon and oxygen, which, with hydrogen, constitute a large portion of the elements of interest to the biologist. The discovery of artificial radioactivity by Joliot and Curie in 1934 led before long to the production of a number of radioactive isotopes which are of value in biological investigations. The past decade and a half have witnessed a swift and enthusiastic application of stable and radioactive isotopes to a wide variety of biologic studies.

The reports of these studies form a voluminous literature. Several reviews (1-7) which deal with various aspects of tracer research have appeared in recent years. The scope of the present discussion is limited to the use of stable isotopes and it is not the purpose of this paper to attempt another review, which would require far more space than is available. This paper is designed to indicate some of the types of investigative problems for which tracers are particularly suited and to present illustrative examples of studies in which stable isotopes have been used.

The discussion is presented in two parts. *I.* Types of problems for which isotope investigation is suitable. *A.* Problems of dilution and transport—the addition of isotopic substance A to a biologic system and the subsequent isolation of substance A and the determination of its isotope

content. *B.* Problems of conversion—the addition of isotopic substance A to a biologic system and the subsequent isolation of substance B and the determination of its isotope content. *C.* Kinetics of biochemical reactions. *II.* An illustration of the use of isotope techniques in the investigation of related problems in one field: the biosynthesis and metabolism of porphyrins.

Problems of Dilution and Transport

Shortly after Hevesy's first experiments with thorium B, one of his colleagues expressed the hope that a tracer would be found which could permit the determination of the fate of individual water molecules such as those in the tea they were about to drink (8). Within a decade Urey's discovery of deuterium and preparation of heavy water had provided an indicator which could help in the solution of such a problem. This type of problem is characteristic of a number of questions which can be answered by a basically simple application of the isotope technique. This application consists in introducing a labeled substance of known isotope concentration into a system and subsequently measuring the isotope concentration of the same substance in the system as a whole or in one or more of its constituent units. One can trace the fate of the labeled substance, *i.e.*, its distribution and localization, and one can measure the time relationships of its distribution. In terms of biological experimentation, this technique is applicable to the study of mechanisms and rates of transfer of biological substances by absorption, secretion or excretion and to the study of diffusion and membrane permeability. The same technique is applicable to the solution of quantitative analytic problems, whether primarily biological or chemical. By determining the dilution in isotope concentration of the labeled substance after its addition to the system one can determine the amount of this substance which is present in the system. In the animal organism precise measurements of a variety of body constituents are made possible, and in a purely chemical system,

the isotope dilution method affords a precise technique for quantitative analysis.

A few examples which are representative of the various applications of this type of tracer work may serve to illustrate the use of the technique. Hevesy and Hofer investigated the rate at which water is eliminated from the human body (8). They administered heavy water to a subject and subsequently measured the deuterium concentration in the urine. By determining the interval between the time of maximal concentration of deuterium in the urine and the time when the deuterium concentration in the urine was one-half the maximal value, they determined $t_{1/2}$, the time required for one-half of the heavy water ingested to be eliminated from the body. This value is nine days. Inasmuch as the biologic behavior of heavy water of low isotope concentration is essentially the same as that of ordinary water, this value for $t_{1/2}$ applies equally to ordinary light water. The average time which a water molecule spends in the body is $t_{1/2} \times \ln 2$, or 13 days.

In the same study, these investigators applied the principle of isotope dilution to determine the water content of the body. After the first day the deuterium concentration in the water of urine may be taken as the deuterium concentration in total body water. This is based on the reasonable assumption that the heavy water becomes completely mixed with the total body water and is handled in the body in a manner indistinguishable from that of ordinary water. The total body water volume is then equal to the product of the deuterium concentration and the volume of the heavy water administered divided by the deuterium concentration in the urine. These calculations yielded a value for total water volume of 43 ± 3 liters or 63 ± 4 per cent of the body weight. This method was modified by Moore (9) who determined the deuterium concentration in plasma one hour after the intravenous injection of heavy water. Complete equilibration of the injected D_2O with the body water was found to occur within one hour; consequently the deuterium concentration in the one-hour plasma sample could be considered equivalent to the deuterium concentration in total body water. By this method, a value for total body water of 47.8 liters or 72 per cent of the body weight was obtained in a normal adult male.

The ability of the tracer technique to follow the transfer of a substance across a membrane has

permitted the study of a number of biological problems which could not be satisfactorily investigated by other methods. Without the aid of isotopes, the measurement of such transfer would depend on the determination of the net change in the quantity of the substance on each side of the membrane. Under physiological conditions, however, little or no net change in the quantity of a great many substances is found despite considerable transfer, for the transfer takes place simultaneously in both directions across the membrane. The isotope technique avoids this difficulty. By introducing a labeled substance on one side of the membrane one can follow the movement of the labeled substance in a system at equilibrium or in a steady state. Inasmuch as the biochemical behavior of labeled units is indistinguishable from that of unlabeled units of the same substance, the transfer of the labeled substance is representative of the transfer of the substance as a whole.

The studies of Gellhorn and Flexner (10) on the transfer of water across the placenta illustrate this application of the isotope technique. D_2O was injected into a pregnant guinea pig, and after ten minutes during which equilibration of D_2O between the various fluid compartments of the mother was known to occur, the fetuses were delivered by Caesarean section. By determining the total body water of the fetus and the deuterium concentrations in maternal and fetal plasma, it was possible to calculate the quantity of water transferred across the placenta per unit time. It was found that the rate of transfer of water across a unit weight of placenta from the 28th day of gestation to term increased about nine times, and that the rate of transfer of water to a unit weight of fetus was parallel to the relative growth rate of the fetus.

The principle of isotope dilution has found ready application to quantitative analytic studies (11-13). Most commonly, a small known amount of labeled substance is added to a system containing an unknown amount of the same substance unlabeled. By determining the dilution in the isotope concentration of the substance isolated from the system, the quantity of substance originally present in the system can be calculated. This technique permits accurate quantitative determinations of a large number of substances for which other good quantitative methods may not be available. In addition, the need for quantitative isola-

tion of the substance is obviated. A pure sample adequate in amount for isotope analysis is sufficient. For accurate results it is essential that the labeled material which is added to the system and the material isolated from the system be pure.

The isotope dilution method has been used to determine quantitatively the optical isomers of amino acids present in amino acid mixtures (14) and to determine the amino acid content of horse hemoglobin (15) and human and bovine albumin (16). Its use has demonstrated that the claim for the presence of large quantities of D-glutamic acid in malignant tumor tissue is invalid (17).

A modification of this procedure has been described by Keston, Udenfriend, and Cannan (18) for amino acid analyses. A mixture of amino acids is treated with a labeled substance, *e.g.*, p-iodophenylsulfonyl chloride labeled with I^{131} . A large amount of the non-isotopic p-iodophenylsulfonyl derivative of the amino acid to be measured is added to the mixture and the compound is subsequently isolated and its specific activity (isotope concentration in the case of stable isotopes) determined. Inasmuch as stable derivatives may be formed with other amino acids in the mixture, the isolated compound may require numerous recrystallizations before purity is established. It should be noted that complete conversion of the amino acid to its derivative must occur if accurate results are to be obtained. This method permits the quantitative determination of microgram quantities of amino acids.

The principle of isotope dilution has been applied in reverse by Bloch and Anker (19) to determine the isotope concentration of a metabolite present in too small an amount to permit direct isolation. Since the amount of isotopic metabolite is also unknown, the addition of a known amount of nonisotopic metabolite (carrier) merely facilitates isolation, but calculation of the original isotope concentration of the metabolite is not possible. If two different quantities of normal carrier are added to separate aliquots of the metabolite solution, however, the two samples of metabolite subsequently isolated will have different isotope concentrations. Sufficient data are then available for calculation of the original isotope concentration. The error of this procedure, however, may be as high as 20 per cent.

Problems of Conversion

Isotopes are particularly suited to the study of conversion reactions. One of the principal types of investigation in intermediary metabolism is concerned with determining the products of the biological conversion of a substance or to determine its biologic precursors. Experiments performed without the aid of isotopes are for the most part of two types: (1) *in vivo* studies in which substance A is administered and the quantity of substance B which is formed in the tissues or is excreted is determined; and (2) *in vitro* studies in which substance A is added to tissue slices or isolated organs and the quantity of substance B formed in the system is measured. Although these types of study have yielded much valuable information, they do not yield conclusive evidence for the determination of biologic precursors or conversion products. *In vivo* studies in which an increased excretion of substance B following the administration of substance A is interpreted as evidence that A is a biologic precursor of B, are open to the criticism that increased excretion of B represents an increased loss of B from the organism rather than increased synthesis and that A is responsible for this effect merely by virtue of its effect on the excretory mechanism. An increased formation of B *in vivo* or *in vitro* following the addition of A does not prove the conversion of A to B but may indicate rather that A is concerned in reactions yielding energy required for the synthesis of B. Furthermore, the absence of increased formation of B following the administration of A does not preclude the role of A as a specific precursor for B. Most biologic syntheses involve a series of reactions. Only one of these reactions may be limiting and principally responsible for the rate of formation of B. If the specific conversion of precursor A to substance B is not this limiting reaction, the administration of A will probably not be reflected by an increased formation of B. Clearly another technique of investigation which offers unequivocal evidence for the specific utilization of structural components of one substance for the biologic synthesis of another is required. Such is the isotope technique.

A striking example of the use of isotopes in the study of conversion reactions is the investigation of the biological synthesis of creatine and creatinine. Bloch and Schoenheimer (20) addressed their attention first to the relationship *in vivo* of

creatine to its anhydride, creatinine. Creatine labeled with N^{15} was fed to rats and subsequently creatine was isolated from the muscles and creatinine from the urine. The N^{15} concentrations in the isolated creatine and creatinine were nearly identical and indicated that urinary creatinine is derived from body creatine. Similar studies were carried out in which after the preliminary period of feeding N^{15} labeled creatine, the rats were kept on a creatine-free diet, during which time the isotope content of urinary creatinine was identical with that of body creatine. This finding indicated that body creatine is the sole precursor of urinary creatinine in an animal on a creatine-free diet. Were any other nitrogenous source present, the isotope concentration in the creatinine would have been lower than that of the body creatine as a result of dilution with non-isotopic nitrogen.

To determine whether the creatine \rightleftharpoons creatinine reaction is reversible *in vivo* as it is *in vitro*, isotopic creatinine was administered to rats and body creatine was subsequently isolated. There was essentially no isotopic nitrogen in the creatine, a finding which indicates that *in vivo* creatinine is not convertible to creatine.

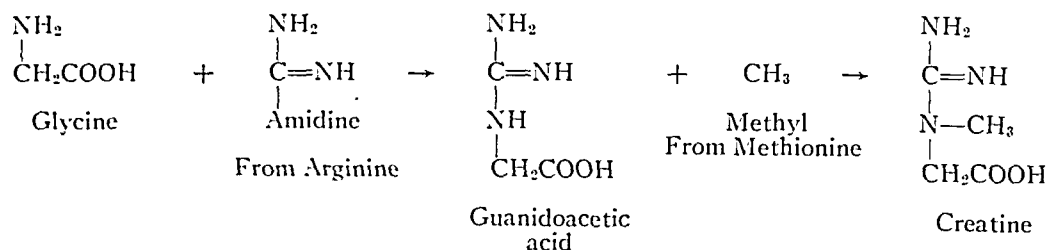
The next step in these studies was the search for the biologic precursors of creatine. Clues to the identity of these precursors were provided by Brand *et al.* (21, 22) who found that patients with muscular dystrophy excrete more creatine after administration of glycine, and by Fisher and Wilhelmi (23) who reported that the addition of arginine to the fluid perfusing rabbit hearts resulted in a quantitative conversion to creatine. In addition Borsook and Dubnoff showed that guanidoacetic acid is methylated to creatine in surviving liver slices and that this methylation is markedly accelerated by the addition of methionine; they concluded that the methyl group of methionine is transferred to guanidoacetic acid to form creatine (24). Simultaneous and independent investigations by Bloch and Schoenheimer revealed that when glycine labeled with

N^{15} was administered to normal rats, creatine with a high isotope content was formed (25). Similarly sarcosine labeled with N^{15} yielded creatine with equally high isotope content. Inasmuch as the ingestion of isotopic sarcosine was shown to lead to the deposition in proteins of isotopic glycine to the same extent as when glycine itself was fed, it was concluded that sarcosine is very rapidly demethylated after its ingestion and that it serves as a precursor of creatine only after demethylation to glycine (26). When guanidoacetic acid labeled with N^{15} was fed, creatine was formed with an isotope content approximately the same as when an equivalent amount of isotopic creatine was administered; clearly, methylation of guanidoacetic acid to form creatine must occur quickly (25).

With the role of glycine as a precursor established and with the demonstration of the rapid methylation of guanidoacetic acid to form creatine, there yet remained the problem of determining conclusively the biologic sources of the various groups in the creatine molecule. Working independently, Borsook and Dubnoff found that kidney slices could form guanidoacetic acid from arginine and glycine (27) and Bloch and Schoenheimer showed that the administration of arginine labeled with N^{15} in the amidine group resulted in the formation of creatine of high isotope concentration (28). By degradation studies the latter investigators were able to show that the amidine nitrogen of creatine is derived from the amidine nitrogen of arginine, and that the nitrogen of the sarcosine group originates from glycine (29).

The final link was provided when du Vigneaud *et al.* found that the administration to rats of methionine labeled with deuterium in the methyl group results in the formation of creatine with a high deuterium concentration (30, 31).

These studies established the immediate biological source of each part of the creatine molecule. The biosynthesis of creatine may be summarized as follows:



Recent studies on the biosynthesis of uric acid offer another example of the use of the isotope technique in establishing the identity of the biologic precursors of an important chemical substance. Sonne, Buchanan and Delluva (32-34) prepared a number of compounds labeled with C^{13} , administered them to pigeons, isolated the uric acid from the excreta, and degraded the uric acid by procedures which permitted separate isotope analyses of each of the carbon atoms in the uric acid molecule. They found that the number 6 carbon is derived from CO_2 , carbon atoms 2 and 8 from the carboxyl carbon of acetate (CH_3C^*OOH) or formate (HC^*OOH), and carbon atom 4 from the carboxyl carbon of glycine ($CH_2NH_2C^*OOH$). Shemin and Rittenberg (35) demonstrated the specific utilization of the amino group of glycine for nitrogen 7 in an experiment in which N^{15} labeled glycine was fed to a human and the uric acid isolated from his urine was degraded. Nitrogen atoms 1, 3 and 9 are apparently derived from ammonia of the general metabolic pool. With glycine established in positions 4 and 7, it remained for Karlsson and Barker (36) using glycine labeled with C^{14} in the α position to show that the methylene carbon of glycine is incorporated into position 5 ($NH_2C^*H_2COOH$). These composite data derived from both avian and human experiments have established the immediate biologic precursors of each component of the uric acid molecule.

Progress in the delineation of the conversion reactions in intermediary metabolism has been markedly facilitated by the use of isotopes. Limitations of space prevent discussion of the many excellent studies which illustrate this application of tracer technique. The interested reader is referred to reviews or representative papers by Wood (37), Evans (38), Gurin (39), Stetten (40), Weinhouse (41) and their colleagues for a more thorough consideration of the use of tracers in the study of conversion reactions.

Kinetics of Biochemical Reactions

Within a few years after the start of their investigations in intermediary metabolism with the aid of isotopes, Schoenheimer, Rittenberg and their colleagues concluded that the conventional concept of the division of metabolic processes into exogenous and endogenous forms was untenable.

Their studies demonstrated that there is a constant interchange of dietary constituents with body constituents and that the body constituents are in a state of flux, of continuous synthesis, degradation, and interchange. They termed this concept of intermediary metabolism "the dynamic state of body constituents." This concept not only comprises an appreciation of the interconversions and synthetic and degradative reactions of body constituents, but it emphasizes the importance of the rates at which these reactions are carried out in the living organism. The kinetics of processes in intermediary metabolism has received relatively little attention, however, as compared with the attention focussed on delineating the pathways of synthesis and degradation. In the future, however, the kinetic aspects of biologic processes may be expected to receive considerably more emphasis in isotope studies. An appreciation of the importance of a metabolic road requires a knowledge of the extent of the traffic on that road. When more than one pathway for the conversion of one substance to another exists, the rates at which the reaction is carried out via the different routes indicate the relative significance of these routes.

Studies in reaction kinetics are pertinent to the investigation of chemical changes in biological fluids and tissues which occur in disease. By determining reaction rates, it is possible to ascertain whether an abnormally high concentration of a substance is the result of an increased rate of synthesis or a diminished rate of degradation, and whether an abnormally low concentration results from a diminished rate of synthesis or an increased rate of degradation.

The considerations and calculations involved in the measurement of reaction rates in isotope experiments have been discussed by Zilversmit *et al.* (42), by Branson (43), and by Radin (44). The principal consideration is the basic assumption of biological isotope research, namely, that isotopic molecules are biologically indistinguishable from non-isotopic molecules. In nearly all cases the additional assumption may be made that the appearance and disappearance of all molecules proceed at random. The simplest type of problem of reaction kinetics is concerned with a biological system in the steady state, *i.e.*, the rate of appearance of the substance under study equals its rate of disappearance and, during the period of study, these rates remain constant. Under these conditions, the total number of molecules, M , of the substance in the system is equal to m , the number of molecules which enter (or leave) the system per unit

of time, multiplied by the turnover time, \bar{T} , the time required for the appearance in the system or disappearance from the system of M molecules of the substance.

One type of experiment in which the rate of synthesis and degradation of a substance may be calculated is that in which a small amount of the labeled substance which is normally synthesized in the system is added to the system and its rate of disappearance is measured. In the steady state the rate of disappearance is equal to the rate of appearance and is consequently a measure of the rate of turnover. The rate of creatine turnover was studied by this technique (45). Adult rats were given isotopic creatine and were then placed on a creatine-free diet. Samples of creatinine were isolated at intervals from the urine. The isotope content of the creatinine was shown in the studies described earlier to be the same as that of the body creatine. The decrease in isotope concentration in the urinary creatinine indicates therefore the disappearance of labeled creatine from the tissues and its replacement by newly synthesized non-isotopic creatine. The curve of isotope concentration in the creatinine declines in exponential fashion, *i.e.*, the decline in isotope concentration at any moment is proportional to the isotope concentration at that moment. Since the slope, K , of the declining isotope concentration curve is equal to $\frac{m}{M}$, *i.e.*, the fraction of the total substance M which disappears from the system per unit time, and since i is the isotope concentration at any time t , then $\frac{di}{dt} = -ki$.

On integration $i = i_0 e^{-kt}$, in which i_0 is the isotope concentration at the start of the experiment. On converting to logarithmic values, $\ln i = \ln i_0 - kt$. A plot of $\ln i$ against t yields a straight line whose slope is k . Accordingly, by serial determinations of the isotope concentration, k can be calculated, and $t_{1/2}$, the half life-time, can be determined from the equation $t_{1/2} = \frac{\ln 2}{k}$. Since $\bar{T} = t_{1/2} \times \ln 2$, the turnover time is also readily calculated. To express the rate of turnover in terms of units of weight per unit of time, M must be determined quantitatively by an independent procedure.

In the case of creatine in rats, the turnover rate was found to be about 0.02 per day. By determining the total body creatine, and the daily urinary creatinine excretion, it was found that the daily creatinine excretion corresponds to about 2 per cent of the total body creatine. The close agreement of these values for daily synthesis of creatine and excretion of creatinine precludes the existence of any major catabolic pathway for creatine other than creatinine formation and excretion.

Hoberman *et al.* (46, 47) have utilized this technique to study the rate of turnover of body creatine in man. In two human male subjects on a creatine-free diet values of 42 and 48 days for the half lifetime of body creatine were obtained $\left(t_{1/2} = \frac{\ln 2}{k} = \frac{0.69}{0.0164} = 42; \frac{0.69}{0.0143} = 48\right)$. When methyl testosterone is administered to the normal subject, the slope of the curve becomes steeper and reflects a more rapid dilution of body creatine (47). By

appropriate calculations it can be shown that the more rapid dilution is the result of an increased rate of synthesis of creatine and an increase in the total body creatine. This study affords direct evidence that methyltestosterone accelerates creatine synthesis.

When it is very difficult to synthesize an isotopic compound whose biologic turnover is to be studied, it may be possible to prepare the compound by biosynthesis and to study its turnover in the same or in another organism. An isotopic compound which is known to be a biologic precursor of the substance is administered to the subject and subsequently the substance is isolated serially and its isotope concentration is determined. In the case of substances in the dynamic state, the isotope concentration rises to a maximum shortly after the end of the administration of the isotopic precursor and then declines exponentially. The upward slope of the curve reflects the appearance of newly synthesized labeled molecules and their replacement of unlabeled molecules formed prior to the administration of the isotopic precursor. The declining portion of the curve results from the disappearance of labeled molecules and their replacement by newly synthesized molecules containing little or no isotope. Inasmuch as the degradation of the labeled molecules may in some cases result in isotopic fragments which can be reutilized for the synthesis of new molecules of the same substance, the rate of decline in isotope concentration may be slower than the actual rate of degradation. This discrepancy is probably not marked, however, for after the degradation of the labeled molecule, the isotopic fragment commonly enters a metabolic pool of similar fragments of lower isotope concentration. A representative sample of this pool is utilized for the synthesis of new molecules. These conditions approximate roughly the replacement of isotopic molecules by non-isotopic molecules and provide a good measure of the turnover rate of a substance in the dynamic state. This technique has been used to study the rates of turnover of serum and antibody proteins in the rabbit in whom it was found that the half lifetime of serum and antibody protein molecules is about two weeks (48). Shemin and Rittenberg have utilized this technique in an extensive study of the interrelationships involved in nitrogen transfer and of the rates of turnover of nitrogen in various tissues of the rat (49).

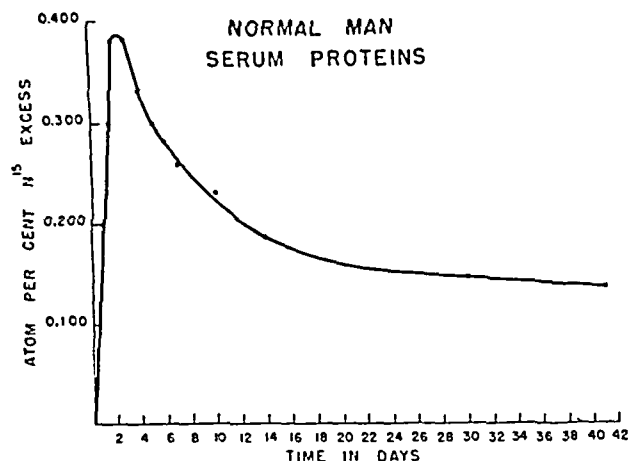


FIG. 1. N^{15} CONCENTRATION IN SERUM PROTEINS AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS

The kinetics of serum protein formation in the human have been investigated by this method (50). Glycine labeled with N^{15} was fed to a human subject for two days and the N^{15} concentrations in the total protein were determined. Figure 1 shows a curve representative of the data obtained in these studies. The maximal N^{15} concentration is reached shortly after the cessation of feeding labeled glycine and thereafter the isotope concentration declines in a roughly exponential manner. The $t_{1/2}$ value for total serum protein is about ten days.

With a $t_{1/2}$ of about ten days, the turnover time, \bar{T} , for a serum protein molecule is $t_{1/2} \times \ln 2$ or about 15 days. The normal human adult male with a total plasma volume of 45 ml. per kilogram of body weight has a serum protein content of about 3 Gms. per kilogram of body weight. Substitution in the equation $M = m \times \bar{T}$ yields a value for m of 0.2 Gm. of serum protein per kilogram of body weight per day. In a normal man of 70 kilograms, approximately 14 Gms. of serum protein are synthesized and degraded daily. It should be remembered that $t_{1/2}$ values are approximations and are somewhat greater than the actual half lifetimes. Accordingly, the shorter actual half lifetimes will be reflected in proportionately greater rates of synthesis and degradation.

A theory and a practical procedure for evaluation of the rate of protein synthesis have recently been described by Sprinson and Rittenberg (51). A small amount of N^{15} labeled amino acid is administered to the subject and the N^{15} content in the urine excreted after the administration of the amino acid is determined. The greater the utilization of the amino acid for protein synthesis, the

smaller the total excretion of N^{15} during the period of study. Mathematical treatment of the data on the cumulative excretion of N^{15} during a period of 48 to 72 hours after ingestion of the isotopic amino acid affords an estimation of the rate of protein synthesis and the size of the metabolic pool of nitrogen. The rate of protein synthesis per kilogram of body weight per day is approximately 0.2 Gm. of nitrogen in adult man. The nitrogen pool, *i.e.*, the nitrogenous compounds which are derived from the diet or from the degradation of body constituents and which are utilized for the synthesis of tissue constituents, was found to be approximately 0.4 – 0.6 Gm. per kilogram in normal man. This procedure should prove to be valuable in the study of a variety of metabolic disorders and in the investigation of endocrine effects on nitrogen metabolism.

The rate of synthesis and degradation of a biologic substance can be determined by still another technique which measures the rate of incorporation of deuterium into the compound from the body fluids. This technique is applicable only to metabolic reactions in which hydrogen, or deuterium, is stably bound to carbon. By maintaining a steady deuterium concentration in the body fluids

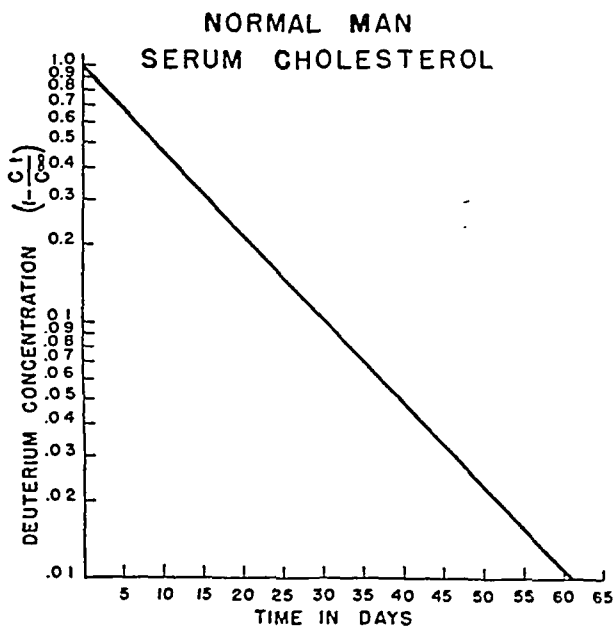


FIG. 2. DEUTERIUM CONCENTRATION IN SERUM CHOLESTEROL PLOTTED SEMILOGARITHMICALLY

C_t = deuterium concentration at any time t . C_∞ = maximum deuterium concentration which is attained at infinite time.

and by serial determinations of the deuterium concentration in the substance under study one can measure the rate at which the substance is synthesized. The maximum deuterium concentration which is achieved in this substance is usually lower than that of the body water since a fraction of the hydrogen atoms of most compounds is not derived from body water. The half lifetime of a substance is the time required to reach 50 per cent of this maximum concentration. Studies on the rate of formation of body cholesterol in the mouse have shown a half lifetime for the cholesterol molecule of 15 to 25 days (52). The rate of synthesis of serum cholesterol in a normal man has recently been investigated by this technique. Figure 2 describes a curve obtained by plotting the isotope concentration against time

$$\left[\log \left(1 - \frac{\text{deuterium concentration at time } t}{\text{maximum deuterium concentration}} \right) \text{ vs. time} \right].$$

The linear curve indicates that the change in isotope concentration is exponential in character and from the slope of the curve the half lifetime of serum cholesterol can be determined. In this normal male subject, the half lifetime is about nine days, the turnover time about 13 days (53).

The Biosynthesis and Metabolism of Porphyrins

The preceding discussion has been concerned with presenting examples of the main types of biological problems which are readily investigated with the aid of isotopes. These studies are for the most part unrelated and are diffusely spread over a number of fields of research. It may be well, therefore, to present an account of studies which illustrate the application of a variety of isotope methods to the investigation of a group of related problems in one field. The studies to be described are concerned with the biosynthesis and metabolism of porphyrins.

These studies were initiated with the finding by Shemin and Rittenberg that glycine is specifically utilized for the biologic formation of the protoporphyrin of hemoglobin (54). When glycine labeled with N^{15} is fed to a human, the hemin isolated from the red blood cells of the subject is found to contain N^{15} (55). Serial determinations of the N^{15} concentration in the hemin describe a curve (Figure 3) (55, 56) which is strikingly different from the type of curve of isotope con-

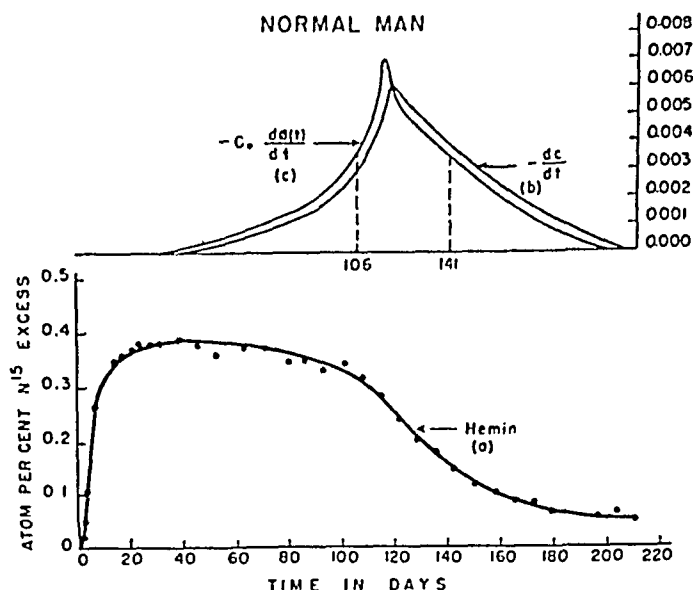


FIG. 3. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS

centration observed in serum proteins after the administration of labeled glycine (Figure 1). The phenomenon observed in the case of serum proteins represents a dynamic process in which protein molecules are continuously being degraded and resynthesized. If hemin were in the dynamic state, even though the red cell itself were morphologically intact, the N^{15} concentration in the hemin would describe a similar curve. The curve of N^{15} concentration in hemin is fundamentally different, however, and cannot be the result of a random synthesis and degradation of hemoglobin in the peripheral blood. The presence of hemoglobin labeled with N^{15} in erythrocytes many weeks after the end of glycine administration must be due to synthesis and incorporation of hemoglobin labeled with N^{15} in the erythrocytes during formation of the cells in the bone marrow. The persistence of labeled hemoglobin in the red blood cells for months after its synthesis in the bone marrow indicates that once incorporated into the red cell the hemoglobin remains with the cell until the cell disintegrates. In brief, the persistence of the labeled hemoglobin reflects the survival or life span of the cells containing the hemoglobin.

The upward slope of the curve of isotope concentration in hemin represents the release into the circulation of erythrocytes containing isotopic hemoglobin and their replacement of cells formed prior to the administration of glycine which contain no isotopic hemoglobin. The maximum iso-

tope concentration is reached and maintained when cells with heme of insignificant N^{15} concentration replace cells formed before the glycine administration. The declining portion of the curve reflects the destruction of cells containing labeled hemoglobin and the point of inflection represents the period of most marked destruction of these cells. The moderately abrupt decline indicates that the heme is not significantly, if at all, reutilized for new hemoglobin formation when the cell disintegrates. If the heme were reutilized significantly, a slower decline in isotope concentration would occur.

It is clear that normal human red blood cells are destroyed as a function of their age and not in indiscriminate fashion. The average life span of the human erythrocyte can be calculated from the curve of isotope concentration in hemin. These calculations are presented in detail elsewhere (55, 56). Values of 127, 120 and 109 days have been obtained in two normal men and one normal woman. Since these subjects were in a steady state, these values correspond to the production

and destruction of approximately 0.8 per cent of the red cells per day. Despite a rather wide range in the ages at death of the red cell population, the time span which encompasses the ages at death of half (the second and third quarters) of the cell population is relatively short. In the two normal male subjects this time span was found to be 28 and 35 days, in the normal female subject 32 days.

This isotope method possesses the unique advantage of making possible the study of the life span and pattern of destruction of the red cells in the same individual in whom the cells are made and destroyed without altering the usual state of the individual. Accordingly, it is well suited to the study of hemoglobin synthesis and red blood cell dynamics in normal and pathologic states.

Polycythemia vera is one of the clinical disorders which has been investigated with the aid of this method (56). The study was performed in an attempt to determine the factors which might be operative in the production of the characteristic abnormality of the disease, namely, the marked increase in the total number of circulating erythrocytes. Theoretically this increase might result from one or both of these factors: (1) an increased rate of hemoglobin and erythrocyte synthesis and (2) prolonged life of the erythrocytes. A patient in a fully developed stage of the disease was given glycine labeled with N^{15} in a dosage equivalent to that used in the normal subjects. Figure 4 describes the curve of the isotope concentration in hemin. The pattern of red cell destruction is normal and the average life span of the red cells is 131 days, a value close to the values obtained in the normal subjects. Inasmuch as the erythrocyte life span is essentially normal, the marked increase in the number of circulating red cells must be associated with an elevated rate of red cell and hemoglobin synthesis. In this patient the rate of synthesis was 2.5 times the normal value.

These findings demonstrate a functional hyperactivity of the blood-forming apparatus in polycythemia vera in a fully developed stage of the disease. It is likely that the development of the polycythemia earlier in the disease is also characterized by increased hematopoietic activity with the maintenance of a normal erythrocyte life span. Various theories have been proposed for the etiology of polycythemia vera but conclusive

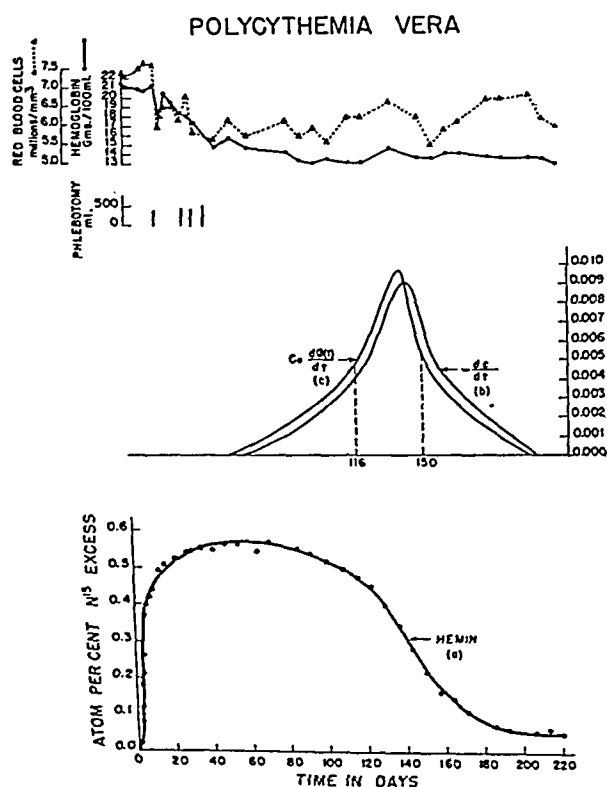


FIG. 4. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS

proof in support of any of them is lacking. The fundamental cause of the functional hyperactivity of hematopoietic tissue remains unknown. The theory, first proposed by Minot and Buckman (57), that polycythemia vera is a neoplasm, appears to be most compatible with available evidence. The persistent bone marrow hyperplasia involving all marrow elements, the development of leukemia in some cases of polycythemia vera and the development of polycythemia in some cases of leukemia suggest that this is a neoplastic process. It appears to be a benign neoplasm which can develop malignant characteristics. If it is a neoplasm, it is an instance of neoplastic growth which is associated with an increase in synthetic activity with no apparent diminution in degradative activity. The fundamental cause, however, remains no less obscure than that of other forms of neoplasia.

Sickle cell anemia is another clinical disorder which has been investigated with the aid of the isotope method (56). A patient with the characteristic findings of this disease received labeled glycine in a dosage similar to that used in the normal subjects. The curve of isotope concentration in the hemin is shown in Figure 5. It is markedly different from the normal. Following a rapid rise to a maximum on the seventh day, the curve declines exponentially. The exponential decline results from a random disappearance of labeled heme from the circulating blood, *i.e.*, the heme is removed from the circulation at a rate which is independent of the age of the heme at the time of its degradation. A curve of this shape could result from (1) a random destruction of the

red blood cells and a consequent loss of labeled heme from the circulating blood; or (2) a random degradation and synthesis of heme in circulating red blood cells which are morphologically intact; or (3) random synthesis and degradation of heme in red blood cells which are themselves undergoing random destruction. The latter two possibilities seemed unlikely in the light of earlier studies which demonstrated that the hemoglobin of circulating erythrocytes is not in the dynamic state. However, when the whole blood of patients with sickle cell anemia is incubated with N^{15} labeled glycine heme labeled with N^{15} is formed (58). This *in vitro* synthesis of heme indicates that the peripheral blood in patients with sickle cell anemia may synthesize heme. The synthesis in the *in vitro* experiments occurs at a rate of only 0.1–0.2 per cent of the red cell heme in 24 hours. If all the hemoglobin in the circulating erythrocytes of sickle cell anemia were synthesized in the peripheral blood at a rate of the same order of magnitude as in the *in vitro* experiments, the hemoglobin turnover would be ten to 25 times slower than that which is actually observed in this case. It would seem that the random disappearance of heme must be due for the most part to an indiscriminate destruction of erythrocytes. If some random synthesis of heme in the peripheral blood of patients with sickle cell anemia does occur, it probably plays a very minor role in the hemoglobin turnover in this disease.

Inasmuch as the erythrocytes of sickle cell anemia are destroyed indiscriminately, they do not have a true life span and their survival is more appropriately measured in terms of their half lifetime. The half lifetime ($t_{1/2}$), *i.e.*, the time required for the isotope concentration to decline from any given value on the declining portion of the curve to one-half that value, is 29 days in this case. The mean survival time, or turnover time ($t_{1/2} \times \ln 2$), is 42 days. With these data and determinations of total circulating hemoglobin and red blood cell volumes, the rates of hemoglobin and red cell production may be calculated. The rates in this patient were found to be nearly three times the normal.

The increased hematopoietic activity is most likely a compensatory response to the markedly reduced number of erythrocytes. The diminished survival time reflects a defect which is in all prob-

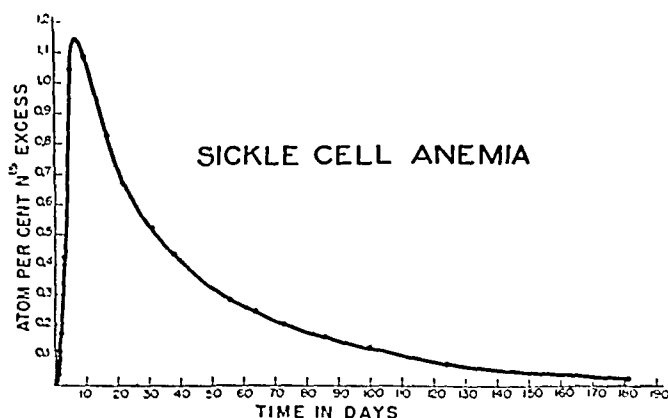


FIG. 5. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS

ability intrinsic to the red cell. This defect, which probably involves the structure of the red cell membrane, may be associated with the sickling process but cannot be ascribed to this phenomenon alone, since the red cells of individuals with sickle cell trait but without anemia are not abnormally susceptible to destruction (59, 60).

Pernicious anemia is another disease process which has been investigated (56). A patient with the typical physical and laboratory findings of the disease received N^{15} labeled glycine. No anti-anemia therapy had been given when the experiment was begun. The curve of isotope concentration in hemin, the red blood cell counts, hemoglobin values, and reticulocyte counts during the course of the study are shown in Figure 6. The isotope concentration in hemin rose rapidly and was approaching its maximum on the 16th day. It was considered inadvisable to withhold treatment longer and liver extract therapy in large

dosage was begun. A satisfactory reticulocyte response and rise in hemoglobin and red blood cell counts occurred.

The rise in hemoglobin and erythrocyte levels was accompanied by a decline in the isotope concentration in the hemin. This decline was anticipated for a dilution of the isotope concentration in the hemin of circulating erythrocytes should result from the release into the circulation of large numbers of new cells formed when the isotope concentration in the body glycine had fallen to a low value. The decline in isotope concentration persisted, however, after the erythrocyte and hemoglobin values approached normal levels and a mere dilution effect should have been minimal. To differentiate a dilution effect from actual destruction of the cells containing labeled hemoglobin, the changes in the total amount of N^{15} in the heme of circulating erythrocytes were determined. It has been shown (61) that the hemo-

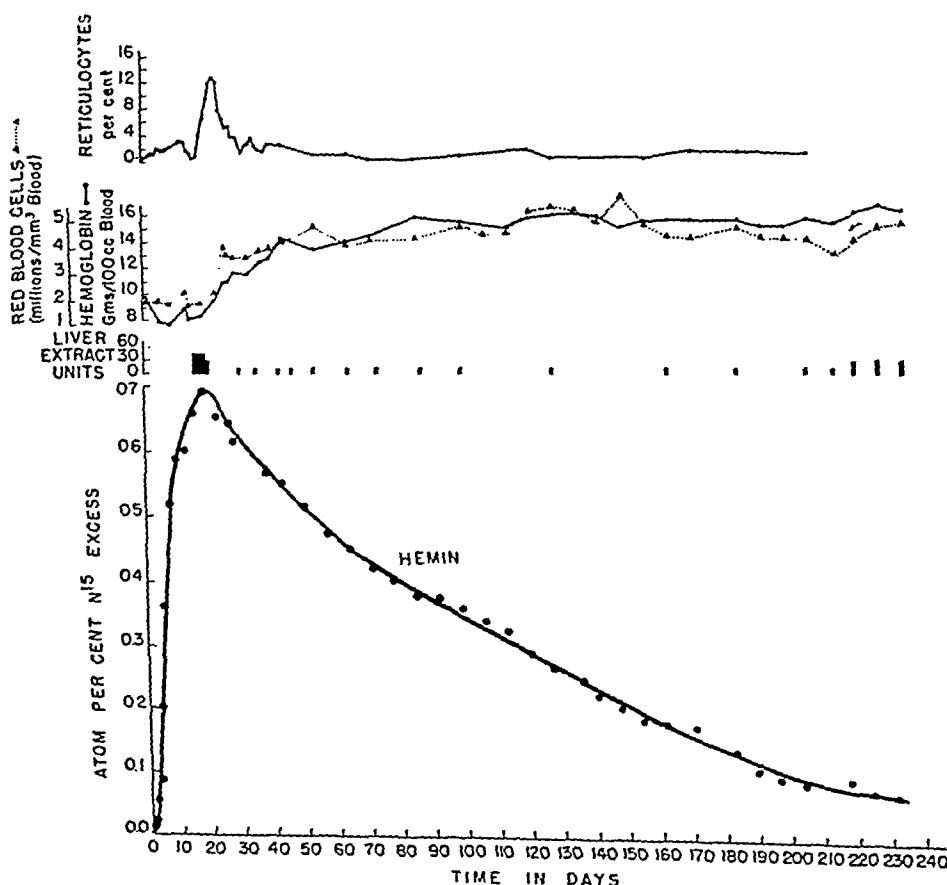


FIG. 6. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS TO A SUBJECT WITH PERNICIOUS ANEMIA

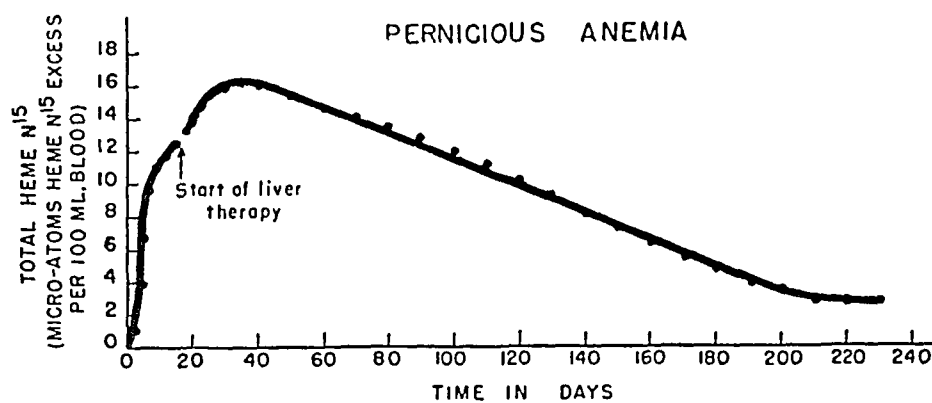


FIG. 7. TOTAL HEME N¹⁵ AFTER FEEDING N¹⁵ LABELED GLYCINE FOR TWO DAYS

globin concentration in the peripheral blood serves as a reliable index of the total hemoglobin in circulation before and after the start of liver treatment in pernicious anemia. Accordingly, a curve representing the changes in the total amount of heme N¹⁵ in circulation could be obtained by multiplying the hemoglobin concentration in the peripheral blood by the isotope concentration in the hemin (Figure 7).

The initial rise in total heme N¹⁵ is similar to the rise in isotope concentration noted in Figure 6. After the start of liver therapy, an additional rise occurs due to the influx of many new cells. Although these newly formed cells contain heme of relatively low isotope concentration, their total number represents a considerable increment in the quantity of heme N¹⁵ in the circulating blood. The maximum value for total heme N¹⁵ is attained about two weeks after the start of liver therapy. If the cells of untreated pernicious anemia enjoyed a normal life span, the curve would have maintained a plateau until the 40th to 60th day and then would have begun to decline. The curve declines, however, in linear fashion almost immediately after reaching its peak. If all the cells were destroyed indiscriminately, the decline would have been exponential. The linear decline suggests that the cell population is mixed, many of the cells being destroyed indiscriminately and others as a function of their age. From the declining portion of the curve it is possible to calculate that the mean survival time of the mixed cell population is 90 days and that the mean survival time of the cells formed prior to liver therapy is approximately 85 days. These calculations are presented in detail elsewhere (56).

With these data and determinations of total hemoglobin and red blood cell values, it can be shown that the rate of production of erythrocytes capable of reaching the peripheral blood is only about 50 per cent of normal. This diminished rate of production and the diminished survival time of the cells in circulation are consistent with the view that in untreated pernicious anemia the erythrocytes are intrinsically defective. The absence of an abnormal hemolytic factor in the plasma of pernicious anemia patients is supported by studies with the Ashby technique (62, 63) which have shown an essentially normal survival of normal cells transfused to recipients with pernicious anemia. There is no marked deficiency in the rate of production of circulating hemoglobin which in this case was 80 per cent of normal.

After treatment with liver extract for one year, the patient was again studied. The findings reveal that the red blood cells are destroyed as a function of age, not indiscriminately, and that their average life span is 129 days, a normal value. Complete restoration to normal red cell dynamics has occurred (Figure 8).

In the untreated state, this patient showed only mild diminution in the rate of production of circulating hemoglobin. But even a normal rate of production and destruction of circulating red cell hemoglobin fails to provide an adequate explanation for the very large amounts of bile pigment which are produced in pernicious anemia. To investigate this and other problems of bile pigment metabolism, the biologic origin of bile pigment has been studied with the isotope technique.

It has commonly been assumed that bile pigment normally is derived almost exclusively, if not com-

pletely, from the degradation of hemoglobin of mature circulating red blood cells. In accordance with this assumption, the appearance of N^{15} in the bile pigment following the administration of N^{15} labeled glycine should reflect the destruction of red blood cells containing labeled hemoglobin. Since in the normal human no significant number of red cells is destroyed for several weeks after their release into the circulation, there should be no significant concentration of N^{15} in bile pigment during the early part of the experiments in the normal subjects. Stercobilin isolated from the stools collected during the first eight days of the experiment in one of the normal subjects was found, however, to have a high concentration of N^{15} (64). This finding suggests that a portion of bile pigment is derived from one or more of the following sources: (1) hemoglobin of red blood cells which are destroyed shortly after reaching the peripheral blood or never reach it and are destroyed in the bone marrow; (2) porphyrins which are not utilized for hemoglobin production; or (3) direct synthesis of bile pigment via a pathway which does not involve degradation of a porphyrin ring. From quantitative considerations it appears unlikely that myoglobin or the respiratory heme pigments serve as a significant source of this portion of bile pigment.

The finding of an additional source of bile pigment formation appeared to offer a reasonable explanation for the discrepancy in untreated pernicious anemia between the very high levels of bile

pigment production and the relatively moderate destruction of circulating red cell hemoglobin. When a similar study was performed in the patient with pernicious anemia, an extraordinarily high concentration of N^{15} was found in the stercobilin isolated during the first several days of the experiment (65). These findings indicate that a very large portion of the bile pigment produced in this disease is derived from one or more of the additional sources which have been suggested.

The investigation of porphyrin metabolism was facilitated by the finding that heme synthesis occurs *in vitro* in the blood of patients with sickle cell anemia (58) and in avian nucleated erythrocytes (66). Since significant heme synthesis is not observed in normal human blood under similar experimental conditions, the *in vitro* synthesis observed in the blood of sickle cell anemia was believed to be due to the presence of numerous immature reticulated cells. On incubation of blood samples from other subjects with hematologic disorders characterized by elevated reticulocyte counts, however, significant heme synthesis was not observed (58). These findings indicated that although the reticulocytes in the blood of sickle cell anemia might be responsible for the heme synthesis, the mere presence of numerous reticulocytes in the blood of subjects with other hematologic disorders does not insure the ability of such blood to synthesize heme *in vitro*. Further studies (67) have confirmed the finding that some blood samples with elevated reticulocyte counts from patients with other blood dyscrasias do not perform significant *in vitro* heme synthesis. These studies have shown, however, that other blood samples with elevated reticulocyte counts from subjects with disorders other than sickle cell anemia may synthesize heme *in vitro*. To investigate some of the problems posed by these observations, the capacity for heme synthesis of reticulocytes in the blood of experimental animals which had no disorder of erythrocyte or hemoglobin formation was studied. The blood of previously normal rabbits in whom reticulocytosis was induced by bleeding or phenylhydrazine hemolysis was incubated aerobically with N^{15} labeled glycine for 24 hours. The N^{15} concentrations in the hemin of these blood samples revealed that approximately 0.3–0.9 per cent of the total heme in the *in vitro* system was

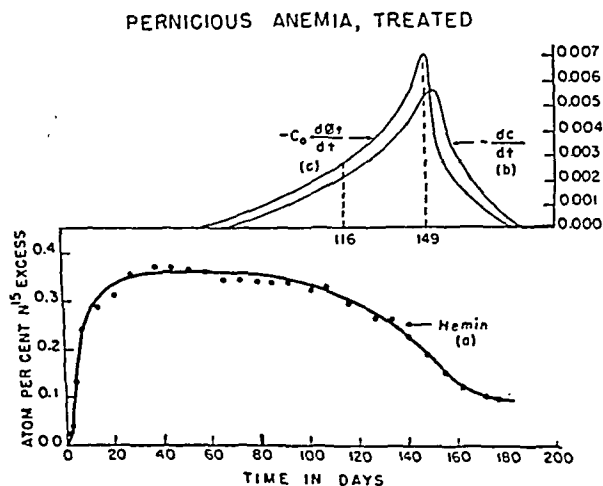


FIG. 8. N^{15} CONCENTRATION IN HEMIN AFTER FEEDING N^{15} LABELED GLYCINE FOR TWO DAYS

newly synthesized during the 24 hour incubation period. These findings demonstrate that mammalian reticulocytes possess a significant capacity for heme synthesis and suggest that some heme synthesis may occur *in vivo* in the reticulocyte after its release into the peripheral blood.

The *in vitro* synthesis of heme by avian erythrocytes has been utilized to determine the precursors which participate in the biosynthesis of protoporphyrin. It has been found, using C^{14} labeled compounds, that both carbon atoms of acetic acid, the carbonyl carbon of pyruvic acid, and the methylene carbon of glycine are utilized in the synthesis of the carbon skeleton of protoporphyrin (68). The utilization of the amino group of glycine for the synthesis of both types of pyrrole rings of protoporphyrin has been demonstrated by finding equal N^{15} concentrations in the two pyrrole types which were obtained by appropriate degradation of hemin formed following N^{15} glycine feeding (69).

The biosynthesis of porphyrins and bile pigment has been investigated in a patient with congenital porphyria who excretes large amounts of uroporphyrin I and coproporphyrin I. Shortly after the feeding of N^{15} labeled glycine, very high concentrations of N^{15} were found in the coproporphyrin I, uroporphyrin I and stercobilin (65). These findings indicate that glycine is specifically utilized in the biosynthesis of porphyrins of the I isomer configuration as well as of protoporphyrin IX which is of the etioporphyrin III isomer configuration. They furnish additional evidence in support of earlier observations (64) that bile pigment is derived in part from one or more sources other than the hemoglobin of mature circulating erythrocytes.

CONCLUSION

Despite the relatively short period of time during which isotopes have been available, tracer techniques have exerted a pervasive influence on biochemical thought and practice. A definitive evaluation of this influence may have to await more extensive development of isotope investigation. It is possible, however, to indicate the two principal developments in biochemical thought which have already occurred and are due in part to the impact of tracer techniques.

Tracer studies have helped to develop a concept which provides insight into the mechanisms of biological synthesis. It has become evident that complex organic substances to a large extent are synthesized not from compounds of similar complex configuration but rather from small compounds of simple structure. The extensive participation of glycine and acetic acid in biosynthetic processes has focussed attention on the biochemical versatility and significance of small simple compounds.

The outstanding contribution of isotope methodology to biochemical thinking has been the development of the concept of the dynamic state of the body constituents. The experimental basis and theoretical implications of this concept, which includes an appreciation of the kinetics of biochemical reactions, represent a major advance toward a thorough understanding of the processes which occur in the living cell.

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DOSAGE DETERMINATION IN THE USE OF RADIOACTIVE ISOTOPES

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INTRODUCTION

As commonly used, most radioactive isotopes are ingested or injected in soluble form and subsequently deposited with a greater or lesser degree of selectivity in various cells and organs. In the event of a single administration, the number of radioactive atoms present at any particular site will increase at first (as they are drawn from the circulation along with their stable isotopes), it will reach a maximum and, as the source of supply becomes exhausted, it will decrease on account of both metabolic turnover and radioactive decay. The latter is accompanied by the release of ionizing radiation, the type and emission rate of which is the exclusive characteristic of the radioelement itself.

The direct measurement of radiation released in this manner in terms of units already established in Radiology is beset with numerous difficulties of experimental nature. Nevertheless when the physical factors of half life and radiation energy, and the physiological factors of uptake and excretion, are known, it is possible, in some cases at least, to make satisfactory estimates of tissue dosage.

The roentgen, as defined by international agreement, applies only to x- or gamma radiation; it can therefore be used for gamma ray emitting isotopes but not for radiation due to primary beta particles. (The roentgen is defined as "that quantity of x- or gamma radiation such that the associated corpuscular emission per 0.001293 gram of air produces, in air, ions carrying one e.s.u. of quantity of electricity of either sign.")

On the other hand if the energy absorbed per gram of air per roentgen (~ 83 ergs) is made the unit of energy absorption for beta rays, it is possible to establish a comparable basis for beta ray dosage. To be sure, in going from air to tissues certain corrections will have to be made because the energy absorption in tissue per roentgen exposure of x- and gamma rays depends

on both tissue composition and radiation wavelength, but in practice, for soft tissues, these corrections are not very large. Hence it is possible to define an "equivalent roentgen" as "that amount of beta radiation which, under equilibrium conditions, releases in one gram of air as much energy as one roentgen of gamma rays." Since the accepted symbol for the roentgen is "r," it is convenient to designate the equivalent roentgen by "e.r." (This is essentially the same unit as the "rep" or "roentgen equivalent physical" of the Plutonium Project.) In the present paper, formulae expressing the relationship between radiation dose and isotope concentrations are presented and their clinical applications discussed by the use of tables containing a considerable amount of pertinent information. Examples of application of the formulae to specific isotopes and definite problems will also be given. For the mathematical and physical aspects of the problem the reader is referred to the literature (1, 2).

CALCULATIONS

Beta Ray Emitters. When a radioisotope emits only beta rays, the dose is essentially confined to the regions containing the material because the range of the beta particles in tissue is only a few mm. Many organs in small animals used in experiments dealing with isotopes emitting high-energy beta rays are not small in comparison to the range of the beta particles. Proper estimate of the dose in these instances is, in general, very complicated, and must be left to the future.²

The total dose D_β , in equivalent roentgens, due to the complete disintegration of a radioelement biologically stable and present in a uniform concentration of C microcuries per gram of tissue is

$$D_\beta = K_\beta C \text{ e.r.} \quad I$$

where

$$K_\beta = 88 \bar{E}_\beta T \text{ e.r. per } \mu\text{cd.}^3 \text{ per gram;} \quad Ia$$

T is the half life of the isotope in days and \bar{E}_β the average energy per disintegration of the beta rays in million electron volts (Mev).

² This problem is somewhat similar to the problem of gamma emitters (*vide infra*).

³ $\mu\text{cd.}$ —microcuries destroyed, i.e., microcuries completely disintegrated in the tissue.

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Doses per hour and per day are respectively

$$\begin{aligned} d_{\beta}(\text{hour}) &= D_{\beta} f_h \\ d_{\beta}(\text{day}) &= D_{\beta} f_d \end{aligned} \quad \text{II}$$

where f_d and f_h are the fractions of the entire quantity of

the isotope which disintegrate per hour or per day respectively. Values of T , \bar{E}_{β} , K_{β} and f_d are given in Table I which refers to beta ray emitters. Z is the atomic number and A the atomic weight of the elements given in the first

TABLE I

Physical data pertaining to calculations of radiation dosage resulting from beta rays and/or very soft x-ray radiation

Element	Z	A	Radiation	T Half life in days	\bar{E}_{β} (MeV)	K_{β} e.r./ μcd . per gram	f_d fraction disintegr. per day	S_{β} μc per kg	Weight per mc in 10^{-3} gram	Maximum range in water (mm)
Group A: \bar{E}_{β} is known to an accuracy of a few per cent										
Na	11	22	β^+ , γ	1100	0.225	22000	$6.3 \cdot 10^{-4}$	7.3	197	2.1
		24	β^- , γ	0.61	0.540	29	0.68	5.1	0.113	6.4
P	15	32	β^- , 0	14.5	0.695	885	0.047	2.4	3.6	8.0
K	19	42	β^- , γ	0.515	1.395	63	0.74	2.1	0.167	19
Sc	21	46	β^- , γ	85	1.117	870	0.008	14.3	30	1.0
V	23	48	β^+ , K, γ	16	0.175	245	0.042	9.7	5.9	2.8
Mn	25	52	β^+ , K, γ	6.5	0.085	48	0.101	20.6	2.6	2.2
		56	β^- , γ	0.108	0.890	8.5	0.998	11.8	0.046	14
Fe	26	59	β^- , γ	47	0.120	496	0.015	13.4	21.3	1.5
Co	27	56	β^+ , γ	85	0.655	4900	0.008	2.6	36.6	7.0
		60	β^- , γ	1940	0.099	17000	$3.6 \cdot 10^{-4}$	16.5	895	0.8
Cu	29	61	β^+ , K, 0	0.142	0.433	5.4	0.992	18.7	0.067	5.5
		64	β^+ , β^- , K, 0	0.53	0.120	5.6	0.73	24.4	0.26	2.6
As	33	76	β^- , γ	1.12	1.170	115	0.46	1.9	0.655	15.7
Br	35	82	β^- , γ	1.5	0.150	20	0.37	13.5	0.95	1.6
In	49	114	β^- , (γ)	50	0.940	4150	0.014	1.7	44	9.4
I	53	130	β^- , γ	0.525	0.270	12.4	0.73	11.0	0.53	4.5
		131	β^- , γ	8.0	0.180	127	0.083	9.4	8.1	2.2
RaE	83	210	β^- , 0	4.85	0.330	141	0.133	5.3	7.85	5.2
Group B: \bar{E}_{β} is less accurately known than \bar{E}_{β} in Group A										
C	6	14	β^- , 0	$2.1 \cdot 10^6$	0.05	$9.2 \cdot 10^6$	$3.3 \cdot 10^{-7}$	32	$23.5 \cdot 10^4$	0.24
S	16	35	β^- , 0	88	0.055	420	0.0079	30	24	0.2
Ca	20	45	β^- , (?)	180	0.10	1580	0.0039	16	62	0.8
Sr	38	89	β^- , 0	55	0.57	2760	0.013	3	38	7
		90	β^- , 0	9000	0.22	$17 \cdot 10^4$	$8 \cdot 10^{-5}$	8	6200	2.2
Y	39	90	β^- , 0	2.6	0.90	200	0.24	2	18	11
Sb	51	124	β^- , γ	60	0.66	3480	0.012	2.4	57	12.3
Au	79	198	β^- , γ	2.7	0.32	76	0.23	5.7	4.1	3.8
Group C: \bar{E}_{β} includes the total localized x-radiation following decay by electron capture										
Mn	25	54	K, γ	310	0.0054	147	0.0022	340	128	
Fe	26	55	K, γ	1500	0.0059	780	$4.6 \cdot 10^{-4}$	280	633	
Co	27	58	β^+ , K, γ	65	0.035	20	0.012	415	29	1.5
Zn	30	65	β^+ , K, γ	250	0.01	180	0.003	185	124	1.2
Group D: \bar{E}_{β} consists of part of the radiation released in the decay by electron capture										
Y	39	86	K, γ	105	0.005	46	0.007	310	69	
In	49	111	K, γ	2.7	0.0058	1.4	0.23	310	2.3	0.01

The values of K_{β} and S_{β} are based on uniform and biologically stable concentrations of radioelements distributed in tissues of linear dimensions large as compared to the range of the beta particles. The sign "0" under the heading "Radiation" indicates the absence of nuclear gamma rays.

\bar{E}_{β} is the average energy per disintegration.

$K_{\beta} = 88\bar{E}_{\beta}T$ is the radiation dose expressed in equivalent roentgens due to beta rays emitted during the complete disintegration of 1 μc of radioelement per gram of tissue.

$f_d = (1 - e^{-0.693/T})$ is the fraction of the entire quantity of isotope which disintegrates in 24 hours.

$S_{\beta} = \frac{100}{K_{\beta} \times f_d}$ is the concentration of radioisotope expressed in μc per kg which will deliver a dose of 0.1 e.r. to tissue during the first 24 hours of exposure.

TABLE II

Physical data pertaining to calculations of radiation dosage resulting from gamma rays

Element	Z	A	Radiation	T Half life in hours	E _γ in Mev			I _γ at 1 cm mr/μc-hr r/μc-hr	K _γ at 1 cm r/μcd	f _d fraction disintegr. per day	μ per cm ×100
					Annihila- tion radiation	Nuclear gamma radiation					
Group A: elements not decaying by electron capture, or x-ray emission following electron capture so soft that it can be treated like beta radiation and hence making no significant contribution to I _γ											
Na	11	22	β ⁺ , γ	26500	0.511(2)	1.30(1)		13.2	500	6.3·10 ⁻⁴	3.2
		24	β ⁻ , γ	14.7		1.38(1)	2.76(1)	19.1	0.40	0.68	2.5
K	19	42	β ⁻ , γ	12.4		1.51(0.25)		1.95	0.035	0.74	2.9
Sc	21	46	β ⁻ , γ	2040		0.90(1)	1.12(1)	11.4	33.5	0.008	3.2
V	23	48	β ⁺ , K, γ	384	0.511(1.16)	0.98(1)	1.33(1)	16.3	9.0	0.042	3.2
Mn	25	52	β ⁺ , K, γ	156	0.511(0.7)	0.736(1)	0.94(1)	19.5	4.4	0.101	3.2
		56	β ⁻ , γ	2.59		1.77(0.3)	2.06(0.2)	9.4	0.035	0.998	2.7
Fe	26	59	β ⁻ , γ	1128		1.1(0.5)	1.3(0.5)	6.55	10.7	0.015	3.0
Co	27	56	β ⁺ , γ	2040	0.511(2)	0.845(1)	1.26(1)	17.95	37.2	0.008	3.2
		60	β ⁻ , γ	46500		1.16(1)	1.32(1)	13.5	900	3.6·10 ⁻⁴	3.0
Cu	29	61	β ⁺ , K, 0	3.4	0.511(1.56)			4.8	0.024	0.992	3.4
		64	β ⁺ , β ⁻ , K, 0	12.8	0.511(0.38)			1.2	0.022	0.73	3.4
As	33	76	β ⁻ , γ	26.8		0.55(0.37)	1.20(0.12)	2.2	0.083	0.46	3.0
Br	35	82	β ⁻ , γ	36		0.547(1)	0.787(1)	15.1	0.79	0.37	3.2
Sb	51	124	β ⁻ , γ	1440		0.6(1)	1.7(0.5)	7.9	16.4	0.012	3.2
		130	β ⁻ , γ	12.6		0.416(0.55)	0.537(1)	13.05	0.237	0.73	3.4
		131	β ⁻ , γ	192		0.080(0.1)	0.363(8)	2.37	0.66	0.083	3.0
Au	79	198	β ⁻ , γ	65		0.40(1)	0.638(15)	2.4	0.22	0.23	3.4
							0.744(1)	0.283(06)			
Group B: elements with x-ray emission following electron capture whose contribution to I _γ is not negligible											
Mn	25	54	K, γ	7450		0.835(1)		4.9+[11]	52	0.0022	3.2
Fe	26	55	K, γ	36000		0.07(2·10 ⁻³)		[10]	—	4.6·10 ⁻⁴	3.2
Co	27	58	β ⁺ , K, γ	1560	0.511(0.3)	0.805(1)		5.7+[7]	12.8	0.012	3.3
Zn	30	65	β ⁺ , K, γ	6000	0.511(0.03)	1.14(0.46)		3.0+[5]	26	0.003	3.0
Y	39	86	K, γ	2530		0.908(1)	1.89(1)	14.4+[3.1]	52.5+[10.3]	0.007	3.2
In	49	111	K, γ	65		0.173(1)	0.247(1)	2.3+[1.4]	0.22+[0.13]	0.23	3.0

The sign "0" under the heading "Radiation" indicates the absence of nuclear gamma rays. The column headed "Annihilation radiation" refers to positron-electron recombination, the column headed "Nuclear gamma radiation" refers to gamma rays originating in the disintegrated nucleus. The numbers in parentheses indicate the number of photons of the particular energy that are released per disintegration.

In Group B, the numbers in square brackets pertain to x-ray emission following electron capture and, because of the short range of the radiation, they should not be used in computing γ-ray dosage. In practice, this type of dose computation is amenable to the simpler formulae used for β-rays (see Group C in Table I).

I_γ is the exposure in roentgens at 1 cm distance in air from an unfiltered point source of 1 mc, for one hour; or milli-roentgens per microcurie-hour (see formula IIIa).

K_γ = 1.44I_γ × 10⁻³ is the number of roentgens, per microcurie destroyed, at 1 cm distance in air from an unfiltered point source.

f_d = (1 - e^{-0.693/T}) is the fraction of the entire quantity of isotope which disintegrates in 24 hours. (T is the half life in days.)

μ = true absorption coefficient of the γ-radiation in water.

column. The last column gives the ranges in water for the most energetic beta particles of each beta ray spectrum for the different isotopes. It should be noted that the column for K_β gives immediately the dose in equivalent roentgens for each microcurie completely disintegrating within a gram of tissue.

Gamma Ray Emitters. When a substance is a gamma ray emitter, the problem of dosage determination is more complicated. The rays released in a given gram of tissue produce only a small amount of ionization there; most of their energy is expended elsewhere along their paths. The problem becomes somewhat analogous to that of interstitial radium gamma ray dosage, except that instead of discrete sources one is confronted with extended ones.

At a given point the *total dose* D_γ, due to gamma rays emitted by the complete disintegration of a radioelement biologically stable and present in a uniform concentration of C microcuries per gram of tissue, is given by

$$D_{\gamma} = K_{\gamma} C g \text{ roentgens}$$

III

where

$$K_{\gamma} = 1.44tI_{\gamma} \times 10^{-3} \quad \text{IIIa}$$

The dose delivered in one day is

$$d_{\gamma}(\text{day}) = D_{\gamma} f_d, \quad \text{IV}$$

f_d having the meaning expressed above. K_γ expresses the number of roentgens at 1 cm distance in air due to the *complete disintegration* of an unfiltered point source of 1 μc; 1.44 × t is the average life in hours, I_γ the dose-rate in roentgens per hour at 1 cm in air from an unfiltered point source of 1 mc. The quantity g in equation III is a *geometrical factor* depending on the location of the given point, the size and shape of the tissue mass containing the isotope, and on the absorption coefficient μ of the gamma rays in tissue. The values of I_γ and K_γ together with other pertinent information for gamma ray emitting radioisotopes are given in Table II.

Safe Tracer Concentration. In the main Tables I and II are self explanatory. Atten-

tion should be directed to column 9 in Table I, headed S_β , which indicates the "safe tracer concentration," that is, the number of μc per kg of tissue weight which will result in a whole tissue dose of 0.1 r the first day, due to the beta rays alone. (The gamma ray contribution will be discussed below.) In the case of a short-lived element, the dose on succeeding days will quickly decrease to the vanishing point, while for long-lived substances it will continue at an appreciable level for some time. This criterion of "safe dose" is decidedly conservative. One might prefer to choose such a level as 1 r in 10 days or some other value based on a longer period of time. This might avoid complications due to various half lives. However, the present familiar idea of safety is based on daily dose; hence that has been used here. It is not to be assumed that larger tracer doses should never be administered. It is sometimes entirely justifiable to use considerably higher doses in diagnostic problems.

Distribution of the Radiation in Tissue. The physical data presented enable the experimenter to calculate the actual dose delivered to a tissue

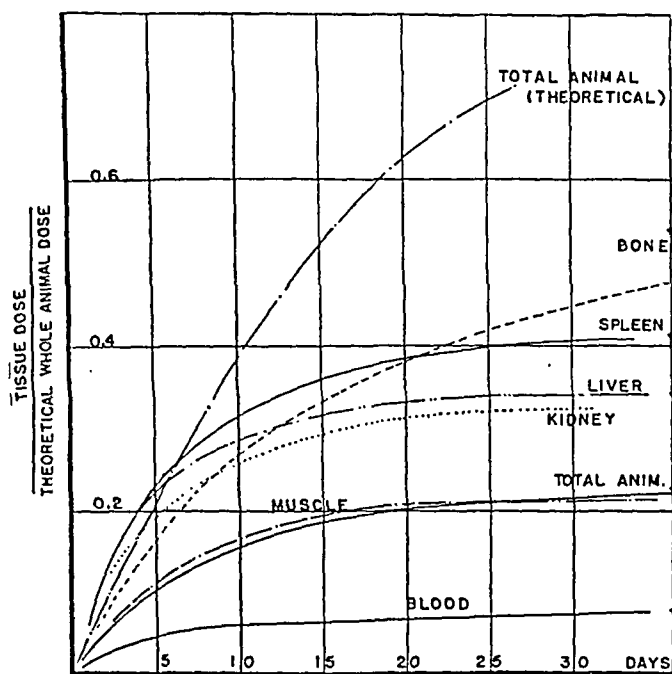


FIG. 1. RADIATION DOSAGE DUE TO THE BETA RAY ACTIVITY OF P^{32} IN DIFFERENT TISSUES IN MICE AS A FUNCTION OF TIME

The points marked on the right margin are the actual tissue doses obtained by extrapolation to infinite time. Theoretical whole animal dose equals K_β times the number of microcuries administered divided by the total weight of the animal.

or individual whenever the isotope concentration C is known as a function of time. This depends on the amount of isotope administered, the species and metabolic state of the test animal, the mode of administration, the chemical form under which the radioelement is given, etc.

Physiological information of this nature is of great importance; the literature shows that a good deal of animal work has been done on the relative uptake of various isotopes by different tissues at different times. As an example, in Figure 1 are shown the relative radiation doses delivered in different tissues in mice injected intraperitoneally with P^{32} in the form of Na_2HPO_4 . The animals were sacrificed at various times after the administration of the material, and the concentration of P^{32} was determined for the various tissues and organs. By adding graphically the daily doses as expressed by equation II it was possible to calculate the dose of radiation received by each tissue as a function of time (3). Similar calculations were made for the whole body assuming uniform distribution. The "theoretical" whole animal dose⁴ after complete disintegration of the isotope was taken as 100 per cent, and all other tissue doses referred to this value. Thus at the end of three weeks, 64 per cent of the theoretical complete dose had been delivered; but all tissues tested, except the bone, had received already practically all their radiation because most P^{32} had disappeared. The liver, for example, had received about 33 per cent of the theoretical uniform dose. At that time the bone had received 39 per cent; but in the bone the dosage increased to 48 per cent at the end of 36 days.

No tissue actually attained the theoretical dose or would have attained it after longer periods of time because of biological elimination. From the curves, the actual tissue dosage per μc of P^{32} thus administered in a mouse of 25 grams can now be calculated. The total animal concentration would be $0.04 \mu\text{c}$ per gram and the "theoretical" total animal dose $D_\beta = K_\beta \times 0.04 = 885 \times 0.04 = 35.4 \text{ e.r.}$ (K_β from Table I). The liver, then, at the end of three weeks would

⁴ By "theoretical" whole animal dose is meant the dose D_β which would have been obtained in the animal after complete disintegration of the isotope on the assumption of uniform concentration in the animal and of total lack of biological elimination.

have received 33 per cent of this value or 12 r; similarly the bone at 36 days would have had $0.48 \times 35.4 = 17$ r.

It is recognized that with P^{32} in mice these determinations are not as accurate as one would wish because the linear dimensions of most organs are smaller than the range of a considerable portion of the beta particles emitted by P^{32} . Similar information in larger animals would yield more reliable figures. Conversely this type of calculation would be fairly accurate even in a small animal, as the mouse, if soft beta ray emitters (H^3 , C^{14} , S^{35} , etc.) were used.

When working with humans it is not possible in general to study concentrations throughout the body and their variations with time by *in vivo* measurements; the extreme concentration of iodine in the thyroid is an exception. For most isotopes information must be obtained from biopsy and autopsy measurements and hence the data are meager; moreover they are scattered through various publications which were often not prepared with this point in mind. Accordingly it has happened too often that the published results lack some essential fact, such as the actual weight of the individual. It is to be hoped that more of this information will be forthcoming. In the meanwhile, it is wise to adopt a conservative attitude in dealing with humans whenever physiological information is lacking. This can be done by assuming that no biological elimination takes place.

Differential Absorption Ratio. To take into account the differences in uptake which lead to differences in dose for the various tissues, it is convenient to express the concentration in terms of a "differential absorption ratio" (D.A.R.). For any tissue, this is the ratio of concentration of an isotope in that tissue to the average concentration in the body (neglecting excretion). Thus under the assumption just made if a particular tissue has a D.A.R. of 1, it receives the same dose of radiation as the average of the whole body. On the other hand, if it has a D.A.R. of 10, it receives ten times as much radiation as the average.

In calculating the safe doses for tracers, it is highly desirable to have some idea of the *range* of the D.A.R.'s. The safe dose is assumed to be 0.1 per day for the entire body; if a certain

tissue has a D.A.R. of 10, it would have received 1 r while the rest of the body received 0.1 r. This might lead to undesirable irradiation of certain tissues or organs from a dose believed safe otherwise.

The procedure outlined above needs clarification. In general, single tissues and the whole body have different rates and modes of elimination; hence the tissue D.A.R. will vary with time. The question then arises as to the time at which D.A.R.'s have significance in determination of tissue dosage. Evidently the D.A.R. will be too low when taken too soon after the isotope administration because the concentrations have not yet stabilized. Likewise adoption of the D.A.R. after several half lives will have little or no significance because most of the isotope has disintegrated. One useful index, whenever the isotope reaches the body tissues through the circulatory system, could be the stabilization of the plasma concentration. No general rule, however, can be given and good judgment is of paramount importance.

Effective Half Life. In instances of extreme concentrations as in the case of *radioiodine* I^{131} administered to humans in the form of KI or NaI, greater accuracy can be obtained. It will be noted that the formula I for the dose, D_b , contains the half life of the element. If the amount of iodine in the gland decreases both by decay and by excretion, the *effective half life* will be less than the physical. Such effects can be determined only by actual measurements. In this particular problem, counts are made at intervals with a Geiger counter in a fixed position over the thyroid gland. If there were no excretion of the material from the gland, these counts should decrease exactly in accordance with the physical decay curve for the iodine; that is, for I^{131} they should be at half their initial value after eight days, one-fourth at 16 days, etc. Actually, they decrease exponentially but more rapidly, half lives as short as two days having been encountered in practice. It is evident that the use of this number instead of 8 in equation I results in a decrease of 75 per cent in the administered dose, an amount which is clinically significant in therapeutic work. It would be now possible to calculate from formula I the dose delivered under normal conditions if the

initial concentration C were known. This can be determined either by actual biopsy or "guessed" by estimate of the gland weight and measurements of the radioactive content of the gland by proper *in vivo* observations.

It is evident from Table I that the actual amounts of the *safe concentrations in tissue* do not vary very greatly; it must be realized that the intervals at which they can be repeated do. A reasonable interval between full tracer doses of any particular element would be four or five half lives, unless the material has been shown to be excreted fairly completely in a shorter period. If no significant excretion occurs, a tracer of Na^{24} could be repeated within about three days, while P^{32} should not be repeated in less than about two months, and Ca^{45} not before two years have elapsed.

It should be mentioned in this connection that the safe doses listed here are calculated on the assumption that the individual is receiving no radiation from any other source. Extensive radiographic or fluoroscopic procedures may result in an appreciable dose of radiation, and such possibilities must not be overlooked.

TRACER ISOTOPES—EXAMPLES

In order to illustrate the use of the material presented in this paper sample calculations will be given for some isotopes commonly used in tracer experiments and in therapy. These will be presented in order of the complexity of their spectra.

Radioactive phosphorus— P^{32} —(Beta particles only)

If the isotope were evenly distributed throughout the body and no elimination took place the values of Table I would be directly applicable. Thus, if $2.4 \mu\text{c}$ per kg gives a dose of 0.1 e.r., an initial radiation dose of d e.r. per day will require a concentration $x \mu\text{c}$ per kg such that

$$2.4:0.1 = x:d,$$

namely:

$$x = 2.4d/0.1 = 24d \mu\text{c per kg}.$$

If a total radiation dose D_β (e.r.) is to be given to a tissue, the concentration in that tissue should be such that

$$D_\beta = K_\beta C \quad \text{or} \quad C = D/885 \mu\text{c per gram}.$$

As already mentioned, the most exhaustive

information on the distribution of this isotope in mice (3) shows that the highest radiation dose is given to bone, followed in order by spleen, liver, kidney, muscle and blood. In general, the limited information on adult humans obtained for the major part from autopsies of leukemic patients (4-9), does not contradict the results in mice, provided that only non-infiltrated tissue obtained from patients given single doses of P^{32} be taken into consideration. Limited data on non-irradiated surgical material are also available (10). The most reliable D.A.R.'s on bone are of the order of unity though wide variations are noted not only among individuals but also among portions of the same bone (5, 10). The absolute value of the D.A.R.'s for human bone—as in the case of mice—seems to bear no detectable relation to the time elapsed between administration and assay; this points to a slow "net" elimination of P^{32} from bone.

The highest D.A.R. reported on human marrow (7, 10) devoid of *leukemic infiltration* can also be taken of the order of unity for the first few days following P^{32} administration. Data on sternal aspiration (7), however, are decidedly lower, being much closer to that of blood. The latter in humans receives "apparent" doses of the order of 20 per cent of the theoretical whole body dose (11). It seems therefore that doses of the order of $2.4 \mu\text{c}$ per kg of body weight can be safely administered, though they may produce wholly transitory but definite biological effects, such as those reported by Low-Beer and Treadwell (12) for concentrations of 6-9 μc per kg of body weight.

If P^{32} is administered intravenously in *insoluble form* the deposition in the liver and spleen is likely to be very marked (13). The authors report 80-89 per cent in the liver (dog and mouse); if this were to obtain in humans the D.A.R. would be:

$$\text{D.A.R.} = \frac{\frac{\mu\text{c in liver}}{\text{liver weight}}}{\frac{\mu\text{c administered}}{\text{body weight}}} = \frac{0.89 \times 70}{1.7} = 36.7,$$

1.7 kg being the weight of the average human liver (14). In this case the maximum permissible concentration would be

$$2.4/36.7 = 0.066 \mu\text{c per kg of body weight}$$

or a total tracer dose of $0.066 \times 70 = 4.6 \mu\text{c}$ of P^{32} for a 70 kg man.

Radioactive strontium—Sr⁸⁹—(beta particles only)

Work on animals (15) indicates that this isotope is *highly concentrated in the bone*. Human material has been reported by Treadwell *et al.* (16) following intravenous injections. Their data point to a maximum D.A.R. in the epiphysis of bone of 13.2, and an unweighted average of 8.3 for bone, mostly in young subjects. A trend of D.A.R. change with time is not apparent. On the basis of this, the tolerance concentration would be, since $S_\beta = 3 \mu\text{C per kg}$, $S_\beta/8.3 = 3/8.3 = 0.4 \mu\text{C per kg of body weight}$.

Radioactive calcium—Ca⁴⁵—(beta particles only)

The relative concentrations of this isotope in human tissue have not been reported to date. Work on mice shows that the D.A.R. for Ca in animal bone is approximately twice that for Sr (15). Hence, if this ratio holds also for humans one should expect a D.A.R. of about $8.3 \times 2 = 16.6$.

It follows from Table I that the tolerance concentration would be, since $S_\beta = 16 \mu\text{C per kg}$,

$$16.0/16.6 \approx 1.0 \mu\text{C per kg of body weight}.$$

It should be noted that if calcium were deposited exclusively in the human skeleton and the distribution were uniform the D.A.R. would be 10 (weight of skeleton = 10 per cent of body weight); nevertheless, the most active parts of the bone concentrate it more and hence the higher value adopted is not unreasonable.

Radioactive sodium—Na²⁴—(beta and gamma radiation)

This isotope of sodium, administered orally or intravenously, is eliminated in very small amounts during times comparable to its half life. Since it is distributed rather uniformly throughout the body (17, 18), computation of beta ray dosage can be made on the basis of D.A.R. = 1. The factor S_β of Table I can be applied directly in the case of small animals where the beta rays account for most of the radiation dose, the factor g in the gamma ray formula being very small. For larger animals and humans, however, the gamma ray contribution cannot be neglected. In this case dosage calculations become more involved.

As pointed out above the contribution of gamma rays to the daily tissue dose is

$$d_\gamma = C_\gamma K f_d g \text{ roentgens where}$$

C = concentration in $\mu\text{C per gram of tissue}$

K_γ = roentgens at 1 cm per μC

f_d = fraction of atoms destroyed in one day

g = geometrical factor which is a function of the size and shape of the tissue volume and the penetration of the radiation.

For purposes of calculation one may assume that the human trunk is a cylinder of radius $R = 20 \text{ cm}$ and height $2Z = 60 \text{ cm}$, and that C is constant throughout. Under these conditions the highest dose is delivered at the mid-point of the axis and g can be expressed with sufficient approximation (1) as $g = 314 - 4140 \mu$ where μ is the absorption coefficient of the gamma rays in tissue. For the gamma rays from sodium (see Table II) the values of μ are 0.0296 and 0.0233 per cm, respectively. An average $\mu = 0.025$ may be assumed for the present calculation and hence $g = 210.5$. Substituting for K_γ and f_d the values from Table II, one obtains:

$d_\gamma = 0.40 \times 0.68 \times 210.5 C = 58.0 C \text{ r per day at the center of the trunk if } C \text{ is given in } \mu\text{C per gram of tissue}.$

Similarly (Table I):

$d_\beta = 29 \times 0.68 \times C = 19.7 C \text{ e.r. per day if } C \text{ is given in } \mu\text{C per gram of tissue}.$

Hence the total dose for the first day is $d_{\beta+\gamma} = 77.7 C$. The total dose $D_{\beta+\gamma}$ can be obtained from this figure. Since 68 per cent of the atoms disintegrate during the first day, 77.7 C is 68 per cent of the total dose. Hence the latter is:

$$D_{\beta+\gamma} = \frac{100}{68} \times 77.7 = 114 C \text{ e.r.}$$

For the tolerance dose of 0.1 r per day,

$$S_\beta = \frac{0.1}{77.7} = 0.0013 \mu\text{C per gram}$$

or 1.3 $\mu\text{C per kg of body weight}$.

Radioactive sodium—Na²²—(beta and gamma radiation)

The computation of tolerance dose follows closely that illustrated for Na²⁴ except for the numerical values involved. Thus, $K_\gamma = 500$; $f_d = 6.3 \times 10^{-4}$; $g = (314 - 4140 \times 0.032) = 181$, since μ can be considered to be 0.032 for the purpose of this approximation.

Hence,

$$d_\gamma = 500 \times 6.3 \times 10^{-4} \times 181 = 57 C \text{ at the center of the trunk and}$$

$$d_\beta = 22,000 \times 6.3 \times 10^{-4} \times C = 14 C$$

$$d_{\beta+\gamma} = 71 C \text{ e.r. per day if } C \text{ is in } \mu\text{C per gram of tissue}$$

$$D_{\beta+\gamma} = 11.3 \times 10^4 C \text{ e.r.}$$

$$S_{\beta+\gamma} = \frac{0.1}{71} = 0.0014 \mu\text{C per gram} = 1.4 \mu\text{C per kg of tissue}$$

or body weight since C is considered constant throughout the body.

This dose ($D_{\beta+\gamma}$) would be delivered over a period of years if there were no excretion of the sodium. However, experimentally the material has a physiological (or effective) half life of the order of one month or less, in normals and in individuals with congestive heart failure (19). In these cases the actual $D_{\beta+\gamma}$ is nearer to 3,000 C. For nephrotics excretion is very much slower, and the dose of radiation would be correspondingly larger.

These two sodium isotopes offer a good illustration of the point made above regarding the repetition of tracer doses. Although the doses of the two isotopes which will give 0.1 e.r. the first day are essentially the same, the total dose from this amount of Na^{24} may be repeated much sooner.

Radioactive potassium— K^{42} —(beta and gamma radiation)

Under the same assumptions of distribution and elimination as in the case of Na^{24} ,

$$K_{\gamma} = 0.0348, f_d = 0.739 \text{ and } g = 198$$

$$d_{\gamma} = 0.035 \times 0.74 \times 198 \times C = 5.2 \text{ C at the center of the trunk, } d_{\beta} = 63 \times 0.74 \times C = 46.5 \text{ C}$$

hence

$$d_{\beta+\gamma} = 51.7 \times C \text{ e.r. per day (C in } \mu\text{C per gram)} \\ D_{\beta+\gamma} = 70 \text{ C e.r.}$$

and finally

$$S_{\beta+\gamma} = \frac{0.1}{51.7} = 1.9 \mu\text{C per kg of body weight.}$$

Radioactive iodine— I^{130} —(beta and gamma radiation)

The distribution of radioiodine in the body is characterized by a *high and relatively stable deposition in normal thyroid tissue* and a low concentration in the rest of the body.

Assays in rabbits and rats (20) show that at various times after administration of iodide, most known radiosensitive tissues show average concentrations of the isotope close to that of blood. Unfortunately no information is available for the marrow. The concentration in thyroid tissue, however, is so markedly different from the rest of the body that the dosage problem assumes different aspects according to whether tracer studies or radical therapeutic procedures are contemplated. Full discussion of the latter is outside the scope of the present paper (21, 22).

In the case of tracer studies it is of interest to calculate the dose to the thyroid itself, since it is very unlikely that the radiosensitivity of any other tissue in the body is such as to overcome the very large thyroid D.A.R. From Table I the beta ray daily dose is readily calculated.

$$d_{\beta} = 12.4 \times 0.73 \times C = 9.1 \text{ C}$$

For the calculation of D_{γ} , from Table II $K_{\gamma} = 0.237$; $f_d = 0.73$. The geometrical factor g is very different from the cases of sodium and potassium in which the whole body dosage was considered. The usual gland (about 25 grams) can be represented by two separate spheres of radius equal to only 1.4 cm and, for this calculation, we may neglect the influence of the radiation from one lobe on the other. In this case $g = \frac{4\pi(1 - e^{-\mu R})}{\mu} \sim 4\pi R$ = 17.6 since μR is very small. It follows that

$$d_{\gamma} = 0.237 \times 0.73 \times 17.6 = 3.0 \text{ C}$$

at the center of the lobe. Hence

$$d_{\beta+\gamma} = (3.0 + 9.1) \text{ C e.r. per day, C being in } \mu\text{C per gram of thyroid weight.}$$

$D_{\beta+\gamma} = 16.6 \text{ C e.r. and } S_{\beta+\gamma} = 0.1/12.1 = 0.0083 \mu\text{C per gram of thyroid weight. For a 25 gram thyroid this would mean } 0.0083 \times 25 = 0.2 \mu\text{C of } I^{130}.$

Radioactive iodine— I^{131} —(beta and gamma radiation)

Following the same pattern used for I^{130} ,

$$d_{\beta} = 127 \times 0.08 \times C = 10.5 \text{ C}$$

and $d_{\gamma} = 0.66 \times 0.083 \times 17.6 \sim 1.0 \text{ C at the center of the lobe; hence}$

$$d_{\beta+\gamma} = 11.5 \text{ C e.r. per day if C is in } \mu\text{C per gram of thyroid weight.}$$

$D_{\beta+\gamma} = 139 \text{ C e.r. (if there is no elimination).}$

As to the tolerance dose, $S_{\beta+\gamma} = 0.1/11.5 = 0.0087 \mu\text{C per gram of thyroid; for the whole gland (25 grams) this would mean } 0.22 \mu\text{C of } I^{131}.$

These doses, which deliver 0.1 r to the gland and less than one per thousand of this to the entire body, are obviously well below the accepted tolerance dose to the *whole* body. They correspond to oral or intravenous administration of 0.2 to 2 μC per patient (to account for excretions of 10 to 90 per cent), and are sufficient for excretion studies as well as for assay of surgical biopsy material. They are, however, insufficient for radioautography with thin tissue sections, for extended studies of blood concentrations and for *in vivo* investigations of iodine retention by thyroid tissue by means of external detectors except, possibly, when scintillation counters are used.

Studies on blood concentration and *in vivo* measurements would require doses of the order of five to 20 times those calculated (thyroid doses of 4 to 15 e.r. in about three weeks, if I^{131} is used, taking into account elimination by the organ, and only .4 to 1.5 e.r. in two days for I^{130}). Autoradiographic material can be obtained only with minimal concentrations of the order of several μc per gram of thyroid tissue (23). It seems therefore that in benign conditions, doses permitting excretion and tissue assays, blood concentration and *in vivo* measurements can be expected to produce no untoward effects, since the radiation is limited for a relatively short time to a very small part of the body which is not particularly radiosensitive. However, in the case of radioautographs, when radiation therapy or total thyroidectomy is not contemplated, every effort should be made to obtain them with the fastest film and low photographic density (23).

At this point it is well to consider that when tracer studies are to be carried out once or a few times on an individual, for diagnostic purposes, the physician may legitimately employ doses considerably in excess of those giving only the radiation permitted for continuous exposure. The diagnostic radiologist does not hesitate to give local exposures of several roentgens, and to repeat these at need. The justifiable dose in such cases must be determined by the clinician responsible for the patient.

The above examples illustrate the fact that no general statement can be made regarding the relative importance of the beta and gamma ray contributions to dosage. In the first place, the relative amount of energy emitted in the form of beta and gamma rays varies from one isotope to another. Also the geometrical factor g , which is a function of both penetration of radiation and size of organ, is likely to vary tremendously with the problem, as in the cases of sodium and iodine.

MISCELLANEOUS FORMULAE

The weight of one millicurie of carrier free isotope can be readily calculated by noting that the weight of an atom of an element of atomic weight A is $(A/N) \times 10^3$ mg where $N = 6.02 \times 10^{23}$, Avogadro's number. Since 1 mc of an isotope contains $1.44 \times T \times 3.7 \times 10^7 \times 8.64 \times 10^4$ atoms (T in days) it follows that the weight of 1 mc = $1.44 \times 3.7 \times 10^7 \times 8.64 \times 10^4 \times 10^3 \times A \times T / 6.02 \times 10^{23} = 7.65$

$\times 10^{-9} \times T \times A$ mg. Values derived from this formula for the various isotopes are presented in Table I, column 10.

It seems of interest to close this paper with an illustration of the weights of radioelements necessary to produce marked radiation effects. This can be done by the following considerations. In order to deliver 1 e.r. to a tissue by means of a beta ray emitting isotope a concentration C is required such that

$$C = 1/88\bar{E}_\beta T \mu\text{c per gram of tissue.}$$

In terms of weight

$$C = 7.65 \times 10^{-12} \times 10^{-3} \times A \times T / 88\bar{E}_\beta T = 8.7 \times 10^{-17} A/\bar{E}_\beta \text{ gram per gram of tissue.}$$

As an example, for P^{32} , $A = 32$; $\bar{E}_\beta = 0.7$ Mev; hence for a lethal whole body dose of 1,000 r the concentration required is $C = 8.7 \times 10^{-17} \times 32 \times 10^3 / 0.7 = 3.98 \times 10^{-12}$ gram per gram of body weight (a total of 0.28 μg for a 70 kg man). Since phosphorus constitutes only 1.14×10^{-2} of the weight of the human body (24) only one atom in 2.85×10^9 atoms of phosphorus need be replaced on the average to obtain such an effect, on the assumption that no elimination takes place.

SUMMARY

When radioactive isotopes are employed either as tracers or in therapy, it is important to be able to determine the radiation dosage. This cannot, in general, be measured, but when the half life, radiation energy, and biological uptake and excretion are known, it can be calculated.

Since the basic information regarding radiation disintegration schemes and energies is scattered through many journals, it has been considered desirable to collect pertinent data. Two extensive tables are presented, for beta and gamma rays respectively, giving half life, radiation average energy, fraction disintegrating per day, and specific dosage data, including the safe tracer concentration, for some 33 isotope elements.

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PERSONNEL PROTECTION IN THE USE OF RADIOACTIVE ISOTOPES

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Artificially produced radioactive isotopes are distributed by the Atomic Energy Commission according to strict rules on account of the dangers inherent in the handling of such materials. Before an individual can obtain radioisotopes, he has to present evidence that he possesses the knowledge, facilities and equipment to meet the minimum standards of safe handling. The Isotopes Division in Oak Ridge, Tennessee, is anxious to help the users in matters of protection and makes available to them articles or pamphlets on this subject, as well as expert advice. It may be assumed, therefore, that the reader is familiar with recent publications on the subject or may get the available information by simply asking for it. Also, a comprehensive report on "Safe Handling of Radioactive Isotopes and Fission Products" is being prepared by the National Committee on Radiation Protection, and will soon be ready for distribution. Accordingly, in the present article no attempt will be made to cover the whole subject, and in fact only a few topics will be discussed, which in the opinion of the writer deserve special emphasis.

The mental attitude of the worker plays an extremely important part in the problem of protection. Most people who have received some cursory instruction, are very careful at the start. Sooner or later a minor "emergency" arises and the individual decides that it is necessary to disregard some rules. The "emergency" might consist simply in not having allowed enough time for the preparation of a sample to be used on a patient at a specified time. Perhaps the surgeon is waiting for the material in the operating room. To save time, the worker then goes through the necessary manipulations without using the protection devices provided for the purpose. For some time thereafter, he might be a little apprehensive and think that perhaps he got too large an exposure. However, unless the exposure was really excessive, he cannot detect any ill effects from it and after a few such experiences he is apt to conclude that the danger of overexposure has been

exaggerated, or that perhaps he is particularly resistant to radiation.

Every worker must be made to realize that radiation injury may not become apparent or serious for many years. This is true even in the case of a single exposure or a few exposures taking place within a matter of weeks. Recently the writer heard of a case in England in which cancer of the skin developed 40 years after the patient had been treated with X-rays in the same region for another ailment. In numerous radiologists, and technicians, cancer of the skin of the hands has developed 15 to 25 years after they started to work with X-rays or radium, although the bulk of the exposure occurred during the first few years when the danger of radiation was not fully realized. The individual worker simply has no way of telling what constitutes a safe exposure from his own experience. By the time that some tissue damage becomes evident to him, it may be too late to do anything about it and eventually serious injury or death may result. Therefore, each worker should accept the permissible limits of exposure set by competent authorities and should strive by all possible means to keep his exposure well below these limits at all times.

In this connection it is well to point out that no matter how careful an individual may be, an occasional overexposure is apt to occur through some accident or through carelessness on somebody else's part. If the person has maintained his exposure well below permissible limits, he is in a better position to escape injury if such overexposures do occur through circumstances beyond his control. Furthermore, the worker should never exceed the permissible limits of exposure on the ground that after some definite date he will no longer work with radiation. Conditions may change and he may decide to continue working with radiation indefinitely. It is not good judgment to acquire a handicap needlessly.

People with considerable knowledge of the subject sometimes get in trouble by attempting to estimate the dosage rate in a region in which they are

anxious to work, on the spur of the moment and under great mental stress. It should be an inalterable rule never to venture into a potentially dangerous region without first surveying it *with the proper kind of measuring instrument known by actual test to be in good working order*. A region in which there is a source of ionizing radiation must be considered to be potentially dangerous, whether protective measures have been taken or not, until proven safe by proper measurements. In other words the worker must not decide that the protective measures are satisfactory by mere inspection, unless he is an expert in the field of protection.

In this connection, some common pitfalls will be mentioned. Lead bricks are usually available in a radioactivity laboratory. With these, temporary protective barriers are built up. Sometimes a single barrier is placed between the source and the part of the room that it is desired to protect. This may or may not be sufficient depending on the strength of the source and the radiation it emits. The lead thickness may be ample for radiation passing through it but radiation emitted in other directions will strike walls, etc., and will be scattered all over the room. More often the container rests on the table and lead bricks are placed around it. Again radiation emitted in the direction of the floor and towards the ceiling can be scattered throughout the room. It is always best to surround a gamma ray source with lead of sufficient thickness in all directions. When temporary barriers are set up, a radiation survey with an appropriate meter should be made to make sure that the dosage rate at all points of the working space is within permissible limits. Adjoining rooms should be surveyed too.

In making a survey for scattered radiation in the case of a source not completely surrounded by lead, it should be remembered that the dosage rate may be highest in regions located at considerable distances from the source. Thus all occupied space in the vicinity of a strong source should be surveyed. Ordinary survey instruments do not respond equally to radiation reaching them from different directions. Hence at any one point it is necessary to take readings with the instrument pointed in different directions to determine the maximum and to establish the cause in case additional protection is needed. It should be remem-

bered that floors and ceilings, as well as the walls, scatter radiation, and the instrument should be pointed downward and upward, too.

The preceding discussion is specifically applicable to gamma ray sources. In the case of materials emitting high energy beta rays (*e.g.*, P^{32}) scattered radiations must also be taken into account. In fact the danger of scattered beta radiation is more insidious because it is often unsuspected. Marked scattering of beta rays occurs *in air*, so the presence of solid bodies in the neighborhood of the source is not necessary to scatter beta rays into a region that might be assumed to be well protected. For example the beam of beta rays emerging through the mouth of an uncapped bottle containing a P^{32} solution, is scattered far beyond its geometric confines. In fact some beta rays from the beam will reach practically every part of a small room. The dosage rate at any one point will depend on the amount of P^{32} in the bottle, and on other obvious factors. In making a radiation survey in this case it is imperative to point the instrument in different directions because usually beta rays can enter the device only through one side, which is provided with a suitable window.

When large amounts of beta ray emitting material are involved, it may be necessary to provide protection against the X-rays ("bremsstrahlen"), produced by the beta rays in the material itself and in the container. The production of these rays is the same as that of X-rays in an X-ray tube, when the electrons impinge on the target. As is well known the higher the atomic number of the target material, the higher the X-ray output. Similarly, P^{32} in a lead container would produce a much higher intensity of X-rays than when placed in a glass bottle. Therefore, in the case of pure beta ray sources, shielding is best accomplished by the use of low atomic number materials. (This is the opposite of gamma ray shielding.)

It is generally assumed that isotopes emitting gamma rays are more dangerous to handle than pure beta emitters (from the point of view of external irradiation). This is obviously true because, for one thing, there is usually beta radiation of not negligible energy associated with the gamma rays. The main reason, however, is that in general the penetrating power of gamma rays is much greater than that of beta rays. Thus only

the superficial tissues of the body (to a depth of at most several millimeters) are irradiated by beta rays, whereas the entire body is traversed by gamma rays. In the case of external sources, eventual production of cancer of the skin and induction of leukemia constitute the principal hazards of exposure to ionizing radiation. With gamma rays both hazards are potentially present, whereas with beta rays (which do not penetrate to the blood forming organs) only cancer of the skin need be considered. Since leukemia is always fatal but cancer of the skin can be cured in a large percentage of cases, it is obvious that exposure to gamma rays is by far the more dangerous. However, this statement, which is true in general, must not be interpreted to mean that there is little danger involved in the handling of beta ray isotopes.

At the present time most university or hospital laboratories handle rather small quantities of radioactive isotopes. Reasonable care is exercised in storing these materials. Therefore, most of the exposure occurs in carrying out some manipulative procedure at close range with rather crude protective devices. Under such conditions the hands are exposed to much higher intensities of radiation than the rest of the body and the principal hazard is tissue damage in the hands that may lead to cancer of the skin later—no matter what kind of radiation is emitted by the material. In this respect and under existing conditions, therefore, the external irradiation hazard is not so different in the two cases (*e.g.*, unshielded equal amounts of P^{32} and I^{131}) as might be supposed. Hence the importance of handling P^{32} with great care also. There is an additional reason for doing so. Most workers handle more than one isotope and the total exposure of the hands from all sources must be considered. In the case of beta ray isotopes it is relatively simple to provide, or even improvise, handling devices that reduce the exposure of the hands to a low level, because the radiation can be completely stopped by light shields. It is, therefore, advantageous to do so, since then exposure to gamma rays (for which adequate protection is more difficult to provide) can be correspondingly greater.

The importance of taking great precautions in the handling of beta ray sources can be brought out best by numerical examples. The data given in

TABLE I

Dosage rates at different perpendicular distances from the surfaces of two radioactive preparations

Distance from surface	10 mc P^{32} solution in 10 cm ² dish (see text)	10 mg Ra tube applicator (gamma rays only) (see text)	Ratio P^{32}/Ra
cm	<i>e.r./hr</i>	<i>r/hr</i>	
0	740	450	1.65
1	300	41.6	7.2
2	112	14.4	7.8
5	31.1	2.77	11.2
10	9.94	0.785	12.7
25	1.80	0.131	13.8
50	0.43	0.033	13.0
100	0.084	0.0083	10.1

Table I will serve this purpose well in that comparison can be made with the more familiar problem of radium protection. First it is necessary to explain briefly how the data were obtained.¹ The dosage rate at the surface of the P^{32} solution (740 e.r./hr²) was determined by means of an extrapolation ionization chamber in which one electrode was the solution itself. By subsidiary experiments, a correction factor was determined to reduce the readings to what they would have been, had both electrodes been made of water. (The amount of phosphate in the P^{32} solution was entirely negligible.) The results are expressed in terms of the number of ion pairs produced per gram of air divided by the number of ion pairs corresponding to one roentgen, that is, 1.62×10^{12} per gram of air. This then is the e.r. as used here.

It should be noted that 740 e.r./hr is the dosage rate at the surface of the solution when it is in contact with the body (*e.g.*, the hand or even a finger),

¹ Details of the experiments and additional data will be published in the near future by the writer and Mr. Norman Bailey.

² In the absence of an internationally accepted unit of quantity of radiation applicable to all types of ionizing radiations, different authors have defined units such as the "equivalent roentgen" (e.r.), the "roentgen equivalent physical" (rep), etc., for dosage purposes. There is some confusion at present as to the definitions and magnitudes of these units, because different effects have been used to establish the "equivalence" with the roentgen. Sometimes the equivalence is based on the number of ion pairs produced per gram of air, at other times on the energy absorbed per gram of air or per gram of tissue. The difference in magnitude of these units is roughly 10–15%, depending on which ones are compared. The meaning of the e.r. as used here is explained in the text.

because the contribution of back-scattered beta rays was purposely included in the measurements. The dosage rates at different distances from the P^{32} source were measured with an extrapolation chamber in which the front electrode (graphited nylon film) had an equivalent thickness of 1.5 mg/cm². The back electrode of thick polystyrene provided essentially the same back scattering as soft tissue. Accordingly, the figures represent the dosage rates that would obtain at the surface of the skin at the different distances from the P^{32} source. In the case of the radium tube the dosage rate at the surface was determined by measurement with a cylindrical extrapolation chamber (1). The other values were calculated and do not include back scattering, which, however, is negligible for short distances.

The dosage rates given in the table are for the following sources: (1) Platinum tube containing 10 mg (10 mc) of radium, with a wall thickness of 0.5 mm and an active length of 12–14 mm, enclosed in a plastic sleeve having an outside diameter of 6.34 mm ($\frac{1}{4}$ inch). (2) "Thick" source consisting of 10 mc of P^{32} in water solution in a polystyrene dish with an area of 10 cm² and a depth of 1 cm (10 cc).

It is evident at a glance that the dosage rates for both sources are very high at short distances. A finger in contact with the surface of the P^{32} solution would receive 740 e.r. in one hour, 12.3 e.r. in one minute, or 0.205 e.r. in one second. A finger in contact with the radium applicator would receive about two-thirds of this dose in the same time. In comparing the values for the P^{32} solution and the radium applicator, the most striking thing is that the dosage rate produced by the former shows a marked increase *relative* to the radium applicator dosage rate, as the distance increases to 25 and even 50 cm. At a distance of 1 cm it is already 7.2 times greater than that of the radium tube and at 25 cm it is 13.8 times greater. This means that at a distance of 25 cm directly over the 10 mc P^{32} solution, the *surface* of the hand would receive, in a given time, the same amount of radiation that 138 mg of filtered radium would deliver at the same distance and in the same time. In other words, at this distance and *insofar as the surface of the skin is concerned*, the 10 mc P^{32} solution is "equivalent" to 138 mg of filtered radium. At 100 cm distance it is equivalent to 101

mg of filtered radium. This shows clearly that distance is less effective in decreasing the dosage rate of high energy beta ray sources than in the case of penetrating gamma rays. It will be seen further that the dosage rate is quite high even at a distance of 100 cm from 10 mc of P^{32} as a thick source—in which case most of the radiation is absorbed within the source itself. If the same amount of P^{32} had been evaporated to dryness in the same dish, the dosage rates for all distances would have been much higher.³ Therefore it is more dangerous to handle P^{32} as a thin dry film or deposit than as a thick source of dimensions comparable to the maximum range of the beta rays. It should be noted in this connection that radium in the form of a pure salt or in solution is very dangerous (more so than P^{32}), because of the large amount of high energy beta radiation which it liberates in addition to the gamma rays.⁴ The comparison is made here with sources such as are commonly used in radiological practice.

It is important at this point to reiterate that the dosage rates given in Table I relate only to the surface of the skin. They are directly applicable to the problem of protecting the skin but they give an exaggerated picture of the radiation hazard as a whole. The comparison with the gamma ray dosage rates of the radium applicator is particularly misleading in this respect. It was made in order to emphasize the importance of protection in the handling of P^{32} in hospital laboratories where familiarity with the handling of *filtered* radium might well lead to overexposure of the hands. One who has handled radium applicators for years might not realize that the skin of the hand would get very much more radiation from P^{32} sources of equal millicurie content. However, the overall danger is much greater in the case of radium because the gamma rays penetrate the whole body and can cause damage, with much more serious possible complications (leukemia). The most energetic beta rays of P^{32} are completely absorbed in 8 mm of tissue, and 2 mm of tissue reduce the

³ Experimental data obtained under these conditions will be published later.

⁴ It is well known that radium taken into the body is much more dangerous than P^{32} . In the present discussion only the danger of irradiation by external sources is being considered.

dosage rate of a thick P^{32} source in contact with the skin to about 10%.

Mention has already been made that in making a radiation survey in a laboratory, the proper kind of instrument should be used. This is particularly true in the case of beta rays. Since the main concern is the dose received by the skin, the instrument should be capable of measuring this quantity. At present, ionization chambers are best for this purpose. Obviously the window that admits the radiation into the chamber must be very thin.⁵ The walls of the chamber should be made of, or lined with, organic material in order that the contribution of scattered beta rays to the ionization current be essentially the same as that of living tissue. The thickness of this material should be sufficient to provide the maximum back scatter. One-half the range of the fastest beta rays to be measured, or approximately $\frac{1}{8}$ inch, is ample. It is desirable to make the chamber small and particularly shallow so that the readings would be representative of the dosage rate at the point of reference. Unfortunately, in the usual survey meters, large chambers are used to provide currents readily measurable by portable instruments. The calibration of these meters then presents a

⁵ The cells at the surface of the skin to a depth of perhaps 7 mg/cm² are considered to be non-reactive to reasonably large doses of radiation since they are practically "dead." A window of this thickness is satisfactory to determine the dosage rate at the depth of the presumably radiosensitive skin cells, when the ionization chamber is properly designed in other respects.

major problem. As a matter of fact, no such instrument can possibly read beta ray dosage rates correctly for all isotopes, all distances, and all shapes and sizes of source. The data of Table I may be used to advantage in calibrating beta ray survey meters for P^{32} sources similar to that described here and at various distances. It should be noted in this connection that the *area* of the source influences the reading considerably. Thus, maintaining both the depth of solution (1 cm) and the concentration (mc/cc) constant, the dosage rate at distances of 10 to 100 cm will increase approximately in proportion to the surface area, when the area is varied from 10 to 40 cm².

Time does not permit discussion of other topics. It may be well to state, however, that the National Committee on Radiation Protection has revised the permissible limits of exposure to ionizing radiation. The new values, of interest in the present connection, that will soon be recommended formally are: (1) For whole body exposure to X- or gamma rays up to three million volts, 0.3 r per week measured in air. (2) For local exposure essentially limited to the *hands only*, 1.5 r per week or 1.5 rep per week in the case of beta rays, in both cases measured at the basal layer of the epidermis, which in general may be assumed to be at a depth of 7 mg/cm² below the surface of the skin.

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BIOLOGICAL HAZARDS AND TOXICITY OF RADIOACTIVE ISOTOPES

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Clinical use of radioisotopes requires careful consideration of their human toxicity, both from the standpoint of the patient and from that of the individuals who must handle the isotopes before administration and the excreta afterwards. One must take into account not only the possibility of acute overdosage but also the potentially serious end-results of the fixation in the body of isotopes, particularly those of long half-life, which may lead to chronic radioelement poisoning.

Such information as we have regarding radioisotope toxicity is largely indirect. Knowledge of the physical characteristics of the radiation emitted by an isotope, combined with information regarding its metabolic fate, enables us to make approximate calculations of tissue dosage. The pathologic effects of a given tissue dosage have been ascertained only in a few instances, using experimental animals; in other instances pathologic consequences of isotope administration are described but the exact tissue dosage has not been established. Translation of these results to man has been attainable only in the case of radium, where it is known that malignant tumors have occurred in individuals harboring less than one-hundredth the tissue concentration necessary to produce comparable results in experimental animals. Yet the catastrophic consequences of radium absorption in a relatively small number of exposed human subjects have resulted in a wholly justifiable conservatism in our attitude towards human exposure to other radioelements.

Units and Dosage Calculations

In the case of external X or gamma radiation, the roentgen (r) is a well-established unit having an exact physical meaning based on the measurement of ion concentration in air. It has the dimensions of energy per unit of mass, and is approximately 83 ergs per gram of air. From this has been derived the rep (roentgen equivalent physical) or e.r. (equivalent roentgen), describing the amount of alpha or beta radiation causing equivalent energy release. Energy release in soft tissue is approximately (but not exactly) the same as in air on a mass basis.

In the case of alpha radiation, it is known that the biological effectiveness differs from that predictable from the roentgen equivalence. This is due to the increased local density of ionization along the alpha ray track. The consequences of this vary (1) but in the case of toxicity to the higher animals the alpha rays are five to ten times as effective as beta rays yielding equivalent energy.¹ Parker (2) has introduced the unit "rem" to signify equivalence to the roentgen unit in terms of mammalian toxicity.

Computation of tissue dosage is most readily made in the case of a mass of tissue infinitely large with respect to the radiation, and with the isotope distributed homogeneously therein. The dosage is then given by the familiar formula.

$$\text{rep/day} = 60 \times E_m \times C \quad (3)$$

where C = concentration of isotope in $\mu\text{c/g}$ or mc/kg , and E_m is the mean energy of the radiation in million electron volts. The mean energy of beta rays from the common isotopes is not always precisely known, but one-third of the maximum energy serves as a fair approximation.

Where the surface of such a mass of tissue approximates a plane, the dosage at the surface will obviously be one-half of that calculated above; this situation applies to the beta radiation at the surface of a thyroid containing radioiodine. Beta dosage from other sources which may be defined in simple geometry can be calculated only in so far as the beta ray spectrum is clearly defined; the author has attempted to do this in relation to mitotic changes around a point source in tissue (4). Further discussion of tissue dosage will be found in the paper in this issue by Marinelli (5).

Nature of Isotope Toxicity

The toxicity of an isotope given in high dosage may manifest itself as an acute reaction resembling acute radiation sickness. This will occur in its purest form where the tissue radiation is homogeneous throughout the body and approaches the lethal dosage (which, for man, is in the vicinity of 500 r in the case of a single exposure to X-rays). This syndrome (6, 7) is a complicated one depending in part on the destruction of the more radio-sensitive cells (particularly of the blood-forming

¹ It is possible that the weak beta emitter, tritium (H^3) will fall in the same category as the alpha ray emitters, due to density of ionization in the very short path of the beta ray.

organs) and on the release of heparinoid substances into the blood (8). Administration of the short-lived Na^{24} , which equilibrates with the body sodium, may be expected to elicit the syndrome of total-body radiation. It is of interest that the consequences of injecting a pure bone-seeking isotope (as Sr^{90}) in high dosage are quite similar (6), suggesting that bone marrow radiation may be a large component in the acute radiation syndrome. The relative resistance of the mouse to acute radiation anemia following Sr^{90} administration may be attributable to the tendency of this species to develop ectopic myelopoiesis in the spleen (9). P^{32} , because of its tissue distribution, may be expected to approximate the pattern of the acute radiation syndrome.

Subacute radiation reactions occur where external X or gamma radiation is given intermittently or continuously over a long period, or where an isotope of sufficient half-life is retained in high dosage. In its pure form, aplastic anemia is the predominant feature (6). Other effects may occur where the tissue distribution creates a special case. The most carefully studied instance is that of the radioelements which occur in high concentration in the liver. Here, after several weeks, necrosis, cell damage, hyperplasia and fibrosis occur and lead to hepatic insufficiency. Where mice or rats have been injected with radiocerium, the thin edge of the liver, in which radiation dosage is relatively small, may undergo hypertrophy while the bulk of the liver shows necrotic changes.

It is the nature of late or chronic radiation changes which sets the limits of permissible exposure. The chronic insult of a long-lived retained radioelement may result in the gradual development of morbid changes punctuated, after a long latent period, by carcinogenesis. Thus, human radium poisoning may result in bone sarcoma where the retained dose is in the order of one microgram, and sarcoma of the liver has occurred several years after administration of colloidal thorium dioxide (10). Experimental investigation, which has been done on short-lived animals, has yielded comparable results but only with much higher tissue dosage due largely, no doubt, to the shorter period of observation.

It must be remembered that dosage does not have to be given over a long period of time in order to result in malignant changes. This is shown,

in the case of man, by the series of bone sarcomas following X-ray dosage of 1500 r and above (11). It appears clearly that external beta radiation of rats is effective in producing skin and subcutaneous malignant tumors in single, as well as daily, dosage (12). Single dosages of 3,000 rep were effective after a considerable latent period (eight to 12 months). Analogous results have been seen following the feeding, either in single or daily dosages, of radioyttrium (13). While tissue dosage calculations are virtually impossible, it is clear that both single and repeated treatments yield carcinomas of the colon. It seems fair to state that in no instance do we have clear information relating the dosage required for carcinogenesis as a function of time-distribution of treatment; in other words, the recovery factor is unknown (14).

"Tolerance" or Permissible Dose

No doubt the most difficult datum to obtain experimentally is the one most in demand by users of radioisotopes, namely, the amount of radiation exposure which may safely be withstood. We do not, in fact, know whether a threshold exists for such measurable evidences of morbidity as shortening of life or cancer, while in the case of genetic changes it seems likely that no threshold dosage exists below which there is no probability of radiation-induced mutation. In the matter of human permissible exposure, we rely upon two basic "tolerance" values: the permissible retained amount of radium has been set at 0.1 μc , since this is one-tenth of the amount known to have morbid consequences, and the permissible daily X-ray dosage has been taken as 0.1 r daily, for similar reasons. The National Committee on Radiation Protection has recently recommended reducing the latter figure to 0.3 r per week. This action takes into account recent experimental evidence which shows that detectable consequences may follow daily administration of dosages below 1 r daily, and also implies that a dosage of 0.3 r will probably differ little in its freedom from consequences whether administered at once or over an extended period. Indeed, the clinical results of a single dosage of 15 r, measured as the lymphocyte response, are on the threshold of clinical detectability.

Permissible levels for radioisotope exposure must, in general, be derived from the two figures

cited above, in combination with experimental work which may relate toxicities of bone-seeking isotopes to radium (as in the data on Sr^{89} to be discussed later) and with calculations of tissue dosage based on what metabolic information is available. Such calculations are described by Morgan (15) and by Marinelli *et al.* (16). Levels so derived must, of course, be accepted with certain reservations: where they depend upon average figures for absorption, the great variation existing even between "normal" human beings must be taken into consideration, and where they depend upon mean concentration in a critical organ, we must be certain whether the radiation is actually homogeneous throughout the organ. Moreover, we must always be prepared for unexpected experimental results. Thus, plutonium 239 was found to be about 50 times as effective as radium, on the basis of physical comparison, in producing acute radiation disease (17), no doubt due to its very heterogeneous tissue distribution, while Sr^{89} has likewise been relatively more potent than expected in bone tumor production.

Justification for Dosage Exceeding the Permissible

As noted above, the permissible dosage of external radiation is expressed as a daily or weekly amount, applied to the whole body. It can probably safely be assumed that most topographic or histologic parts of the body can receive larger dosages, as in the case of exposure of the hands. Consideration of the methods used in deriving permissible dosages will show the rather general impossibility of deriving data applicable to various parts of the body.

It also seems that a single dose in excess of the daily permissible level can be defended under some circumstances; for example, much routine radiography results in dosages exceeding daily or weekly "tolerance."

With firmly deposited isotopes of long half-life, the daily dose is necessarily continued after deposition. The tendency in this case has been to calculate the amount, giving the maximum permissible level to the critical organ at the height of its concentration there.

The foregoing discussion applies to permissible levels of radiation exposure for normal individuals who receive their exposure industrially or profes-

sionally. Various ranges of exposure may be justifiable, depending upon the nature of the population or upon the reason for administration. They are indicated below, in ascending order of exposure level.

a. The large population: Because of the statistical nature of the genetic sequelae of the lowest dosage levels, it is probably correct to consider that a large population should not be permitted to become exposed to a level of radiation comparable with that which can be justified in special cases. It is likewise possible that tumor development may be slightly enhanced by dosages under "tolerance," but no evidence on this point is available. In the matter of recessive mutations, the size of the exposed population will, of course, determine the probability that, with random mating, the same recessive will occur in both gametes in a later generation. It is, however, probably unnecessary to prescribe levels less than one-hundredth of "permissible," since this level approaches that of the cosmic radiation and the radioactivity normally present in the body.

b. The small, professionally exposed population: It is for such populations that the permissible levels have been applied. Although a possibility of minimal effect, perhaps not detectable, is not ruled out by our present knowledge of the radiobiologic effects of small dosages, it will be recalled that various other industrial and other hazards exist which, again, are merely reduced to the imperceptible level. No low-level hazard has been as carefully studied as that of external ionizing radiation.

c. Clinical investigation and diagnostic procedures: Where such a reason exists for isotope administration, we may fall back on the precedent of diagnostic radiography. Thus, a total dosage of beta and gamma emitters between 1 and 10 rep to the critical tissue may be justified, if not repeated, in the case of certain isotopes of short half-life whose metabolic fate is well understood, *e.g.*, Na^{24} , K^{42} , I^{131} , and possibly P^{32} . For the present, the bone-seeking isotopes in general, as well as those whose metabolic fate is in doubt (C^{14} in particular), should not be used in this category. Isotopes in a borderline class include Fe^{55} , Fe^{59} , Ca^{45} , and the long-lived isotopes Na^{22} and Cl^{36} ; with these, somewhat more caution is indicated than with the shortest-lived radioelements. As examples of actual practice, Na^{22} has been used in

normal subjects (18) in such a way as to yield somewhat less than 1 rep total integrated dosage ($90 \mu\text{c}$ in man); Fe^{55} and Fe^{59} have been used in normal blood donors (19) in such dosage as to yield a maximum of 0.1 rep per week in blood. P^{32} has been given to human subjects to establish its turnover in tumors, including operable tumors. In one recent study, $1 \mu\text{c}$ per pound body weight was used and it was estimated that the total-body irradiation would not exceed 1.5 r (20) (integrated over time and over the body volume). The attitude toward I^{131} , on the other hand, has been somewhat more liberal, presumably because this isotope is virtually confined to the thyroid. Thus, for diagnostic purposes, 20 rep per day to a total dose of 230 rep has been given as calculated thyroid dosage (21).

It is clear that improvements in technique of measurement of isotopic radiation will greatly benefit work in this area, since many studies will be brought within the limits of reasonably safe operation. As it is now possible actually to estimate naturally occurring C^{14} within 5–10 per cent (22), this is an important direction in which clinical isotope work can find new opportunities.

d. Treatment of disease where other therapy is available: In this range, dosage must necessarily far exceed that required to produce visible changes in the organism, since changes are being sought. The principle of calculated risk is therefore paramount, as indeed it is in all clinical therapy and especially in surgery. There are, however, certain pervading differences. In the first place, the risks in surgical and pharmacologic therapy are mainly immediate, easily observed and soon calculable; whereas in isotope therapy the most important risks may be disclosed by the passage of a considerable amount of time, as shown by the results of radium absorption and by the late consequences of the use of colloidal thorium dioxide (even where used for diagnostic purposes) (10). Secondly, there are certain unknown factors which make calculation of risk, to an order of magnitude, impossible. We do not know the relation between latent period before carcinogenesis and the lifespan of a species, or the relations between the probability of tumor development and the volume or type of tissue irradiated or the time distribution of radiation. On a more basic level, we understand next to nothing about the mechanism of radiation

carcinogenesis or the meaning of the shortening of life after total-body irradiation.

In two instances, there is a fairly large backlog of experience in therapy of disease with radioisotopes. These are the treatment of benign thyroid disease (especially exophthalmic goiter) with radioiodine and of leukemia and polycythemia vera with P^{32} . Therapy of polycythemia vera has yielded results similar to those of X-ray given to the whole body, and is logical inasmuch as bone marrow receives a larger dosage than the integrated total-body dose. One mc administered to a 60 kg man yields an integrated total body dosage of about 15 rep, neglecting loss by excretion, at a rate less than 1 rep per day; the bone marrow, spleen, and lymph nodes may receive two to three times as much. Thus, dosages of 1–5 mc correspond to total-body X-ray treatment ranging between 15 and 75 r, or somewhat more to bone marrow. The time distribution of the radiation probably makes it somewhat less toxic (and less effective) than X-ray given according to usual patterns of treatment.

Although dosages of this sort are in accord with regular practice in radiation therapy, it is possible that deleterious results may occur. This has been suggested by Hall and Watkins (23) in whose series of P^{32} -treated cases a number of acute leukemias arose. Because of the frequency of chronic leukemias and leukemoid states arising in the course of the disease, it is difficult to evaluate these results until further information is available. Our own observations (24) show that P^{32} is leukemogenic to mice, but that the effect is not strikingly different from that of total-body X-ray in equivalent dosage.

In treatment of thyrotoxicosis, I^{131} has been used in relatively high thyroid dosage by a number of clinical groups. This question has been discussed recently by Nickson (25). Dosage of 200 or more microcuries per gram of thyroid are frequently used, with a resultant total radiation dose in excess of 10,000 rep. The actual dose has been estimated to be as much as 26,000 rep with $200 \mu\text{c/g}$ (26), but is difficult to estimate, since it depends on the rate of loss of iodine from the treated gland. If the dose to the tissues immediately surrounding the thyroid is one-half to one-fourth of this, it appears that the adjacent connective tissue receives thousands of rep. This is

in the range of dosage by X-ray which has resulted in bone tumors in man (11), and it will be recalled that 3,000 rep of external beta radiation to rats from P^{32} has resulted not only in epitheliomas, but in subcutaneous sarcomas as well (12). It is, therefore, within the bounds of possibility that carcinogenic effects may occur from dosages as used in the treatment of thyrotoxicosis, although it is encouraging to note that no reports of such sequelae have appeared after several years' clinical experience.

An intermediate group of diseases exists, namely the leukemias and allied disorders, in which certain isotopes (Na^{24} [27], P^{32} , As^{76} [28]) are used in place of total-body radiation for similar palliative purposes. In all of these instances the net effect of the radiation is either equivalent to that of total-body X-ray, or it is distributed in favor of bone marrow and blood-forming organs. Since the peripheral blood picture gives indication both of toxicity and of therapeutic effectiveness, it is useful in monitoring treatment. It should be recalled that the same general remark applies to the use of radiomimetic chemicals, *e.g.*, the alkylamines.

e. Treatment of hopeless malignant disease:

Here it would seem justifiable to use whatever means offer some chance of palliation or cure, or of gaining information to that end where this can be done without detriment to the patient. In the case of metastasized malignant disease of the thyroid, it would seem proper to treat every patient with I^{131} in order to determine the response. Although certain histologic types have been found not to concentrate iodine (29), it has been shown that the capacity to store this element may be enhanced by thyroidectomy or administration of thyrotrophic hormone. Dosages have been large; in one case (29) where the dose to the normal thyroid is stated, the gland should have received 36,000 rep on the first day (assuming immediate uptake) to an ideal total (barring subsequent loss) of 430,000 rep. Treatment in this order of magnitude has been possible only in the treatment of thyroid disease. Dosage to metastases in this case was $\frac{1}{70}$ of that to thyroid (*vis.*, about 6,000 rep).

In breaking new ground, the long-lived isotopes will be tested. The possibilities of C^{14} and H^3 in organic compounds are manifold, and es-

entially no exploration has been done at the present time. The use of Sr^{90} has also been suggested. Because of the known chronic toxicity of the latter and the potential hazard in the use of other long-lived isotopes, it will be essential that cases be selected on the basis of inoperability. It may be suggested that all patients be chosen (1) as a result of biopsy from a metastatic lesion, or a primary lesion deemed inoperable or (2) after consultation with a specialist in this particular field. There are few physicians who have not, on some occasion, misjudged the prognosis of a clinical situation resembling inoperable cancer. It has also been advocated that such treatment be given only after exhaustive experimental study. This is desirable in cases where the toxicity of the isotope or the isotopic compound is unknown, but it should be recalled that the variety of human cancer is greater than that easily available in laboratory animals, so that the appropriateness of the treatment for a given tumor type may need to be evaluated by tracer studies in patients, or (for C^{14}) by use of the stable C^{13} .

As mentioned before, the short-lived isotopes, and especially those yielding hematologic changes as readily as total-body X-ray, may be used with the same precautions as are used with external radiation or chemotherapeutic agents, as far as the patient is concerned. It will be seen that the short-lived isotopes may be intense radiation sources and require special care during preparation and administration.

Toxicity of Bone-seeking Elements

In order to gain some quantitative expression of the carcinogenic action of radioactive substances which might have general meaning, large series of animal experiments were set up in this laboratory using Sr^{90} in rats and mice. It was hoped thereby to observe the pattern of bone tumor induction (as pilot experiments had shown [30]) by a very potent carcinogen the effectiveness of which was due to beta irradiation of bone. A preliminary discussion of these experiments was included in our earlier report (14). The data are still incomplete pending histologic verification of the gross pathologic findings. One of the strains of mice used has shown an unexpectedly high incidence of bone lesions of various sorts, including benign tumors.

Experimental: The mice used in these experiments were females of the Carworth CF-1 strain and male and female ABC hybrid mice obtained from the Roscoe B. Jackson Memorial Laboratory. These mice were chosen because of their ready availability in large numbers at the time the experiments were begun (1945-6) and because they had no specialized tumor characteristics which might obscure the study of the general tumor hazard, except for a high incidence of spontaneous lymphoma and lymphatic leukemia. A few high-lymphoma C58 strain mice obtained through the kindness of Dr. E. C. MacDowell were also used.

The animals were housed in an air-conditioned animal farm to permit regulation of temperature (*circa* 23° C.). Triethylene glycol vapor was used to minimize the possibility of air-borne cross-infection. The mice were received in lots of over 600, equally divided among the three permutations of strain and sex. One-fifth of the total experiment, including aliquots of the dose levels used, was set up upon receipt of each shipment, and injected at $42 \pm$ seven days of age. Each component group of animals remained caged together throughout the experiment. It was found that certain cages of animals suffered from infectious disease, but cross-infections to nearby cages were rare. The commonest infectious diseases were pneumonia (with intracellular pleuropneumonia-like organisms) and intestinal or generalized infections due to *Salmonella* and *Pseudomonas*.

Since the acute lethal dose (30-day LD_{50}) for Sr^{90} in mice was found to be 8 $\mu\text{c/g}$ body weight, the highest single dosage used was 5 $\mu\text{c/g}$, and lower dosages were employed, differing by factors of approximately 2, down to 0.05 $\mu\text{c/g}$. A parallel series was given monthly dosage with one-fifth of the above amounts in such a way as to bring the dosage to the same level each month. Increasing numbers of mice were used in the lower dose levels in order to maintain statistical significance, if possible, where tumor incidence would be less; the design of the experiment is shown in Table I.

All mice were observed daily; those showing evidence of serious disease were followed with particular care and sacrificed when *in extremis*. By adequate clinical observation it became possible to salvage over 95 per cent of the fatally ill animals before death. Complete autopsy (except for the central nervous system) was performed,

sections were taken where considered necessary, and the carcasses preserved in 4 per cent formaldehyde. Roentgenograms were made in all cases at or after death.

RESULTS

Survival was definitely shortened at the higher dosage levels, as might be expected. This is illustrated graphically in Figure 1, in which a separate family of curves is shown for survival of the groups injected singly and monthly. Because of the obvious similarity among the types of mice used with reference to longevity and bone tumor genesis, they have all been pooled in assembling the data in preliminary form. The last survivor in the ABC female control group died 895 days after the beginning of the experiment (about 937 days of age) and the last CF-1 female died after 876 days (about 918 days of age). The ABC male group in the experiment was shorter-lived, falling (for the last 5 per cent of survivors) about 100 days behind the females of the same strain.

Even at the highest dosages, no "acute" radiation deaths occurred during the first month. The earlier mortality among these mice is not ascribable to any single cause. In addition to bone tumors, aplastic anemia and myeloid metaplasia (the latter probably a compensatory response) (9) were commonly observed at autopsy, and graying of hair, which is a frequent subacute sequela of total-body radiation, was noticeable. Of the mice receiving 5 $\mu\text{c/g}$ and surviving 200 days, 35 per cent died before 250 days without bone tumors and an additional 14 per cent, with bone tumors; of the controls, 5 per cent of the 200-day survivors died in the same period.

Incidences of tumors, other than those arising in or near bone, have failed to undergo changes attributable to the treatment. A small apparent increase in lymphomas in the heavily-treated CF-1 and ABC mice has been shown to lack statistical significance.

Tumors in or near bone were of several types; osteogenic sarcomas predominated, but other malignant tumors of mesenchymal and endothelial origin have been observed, and for statistical purposes have been included with those of osteoid origin. Multiple primary tumors occurred with an entirely random frequency, their incidences conforming well to the Poisson distribution. Thus, multiple tumors are more frequently seen as the

TABLE I
Design of large mouse experiment with Sr^{90}

Dose	Single injection	Monthly injections	Dose
$\mu\text{c/g}$	number of mice	number of mice	$\mu\text{c/g}$
5	45	45	1
2.5	60	60	0.5
1	90	90	0.2
0.5	150	150	0.1
0.25	240	240	0.05
0.1	240	240	0.02
0.05	240	240	0.01
Control	480	480	Control

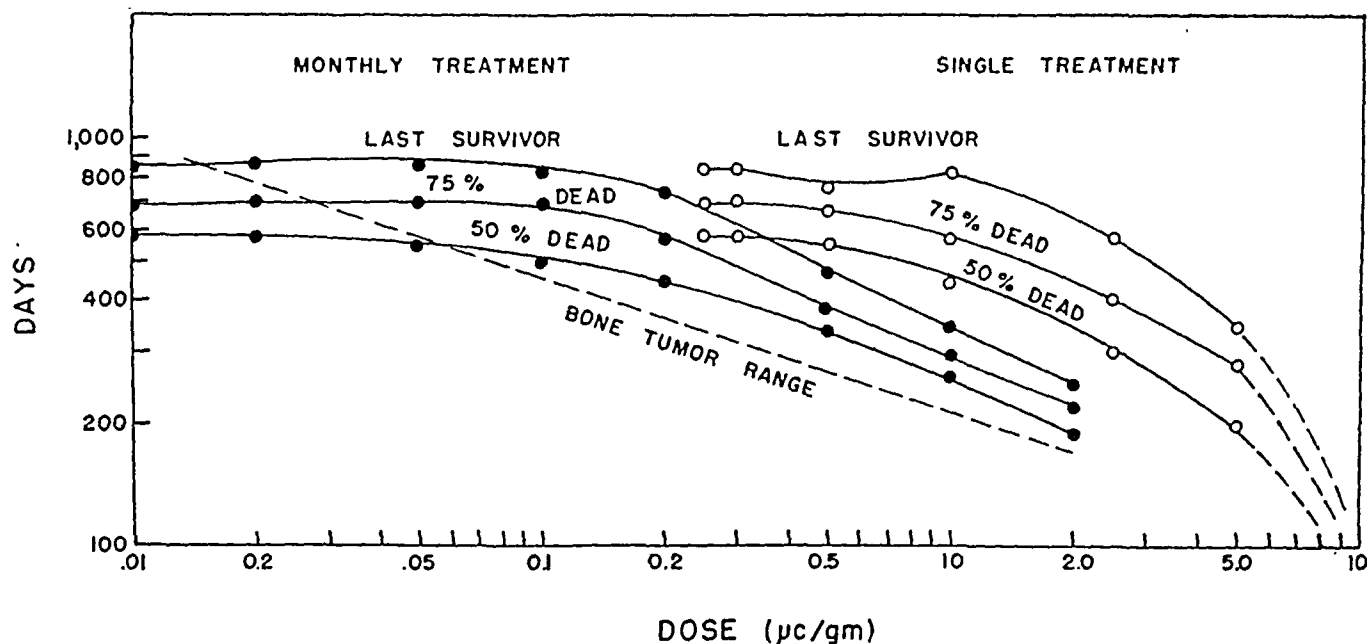


FIG. 1. CHART SHOWING SURVIVAL OF MICE GIVEN VARIOUS DOSES OF Sr^{90}

Open circles represent single injections; closed circles, dose given monthly.

dosage increases. It may be postulated from this that one tumor, already present, does not suppress or stimulate the genesis of others.

A very striking feature of treatment with the higher dosages is the sharpness of the latent period, at the end of which tumors appear rapidly. It seems clear that the latent period cannot be explained (where a single dose is given) by the time required for a tumor to develop from a single cell if the cellular growth rate is constant, unless the tumor is formed some time after the greater part of the radiation has been delivered. As stated earlier, the prolonged latent period is characteristic of radiation carcinogenesis.

In attempting to understand quantitatively the process of carcinogenesis, the data have been plotted in a variety of ways. The simplest function was seen where the tumor morbidity rate was plotted directly against time, in mice given continuing dosage. These data are shown in Figure 2. The ordinates represent the probability that a mouse in a given dosage group will develop a tumor (or possess a tumor at a certain early stage of development) on a given day, and this figure is obtained by calculating, for 40-day overlapping time intervals, the proportion of mice which will die with bone tumors within 40 days. This figure, divided by 40, gives the daily expectancy, or morbidity rate, P_t . The time scale is adjusted to the beginning of the 40-day period

before death, and therefore represents tumors at about the time they are first visible, or somewhat before that time.

It will be seen that this function gives essentially a straight line cutting the horizontal axis at the "latent time"; the slope of the line being proportional to dose, and the latent period varying gradually with dose, perhaps as a logarithmic function. The straight lines have been drawn in accordance with an empirical formula:

$$P_t = Kd (t - t_e)$$

where $(t - t_e)$ represents time after the end of the latent period, and d is proportional to the dose, where the monthly dosage is expressed in $\mu\text{c/g}$, and time in days, and K is taken as 1.4×10^{-4} . The latent period may appear, on inspection, to vary with the logarithm of the dosage, or with an inverse root of the dosage (approximately the square root). The latter function is indicated, quite qualitatively, as a broken line in Figure 1, and shows graphically the impossibility of producing tumors with low dosages in short-lived animals.

It is significant that the accumulated number of tumors at any time after the end of the latent period is a function of $t \times P_t$, and hence of the square of time. In this way, it is seen that the low dosage required to induce radium tumors in man is consistent with the longer period in which tumors

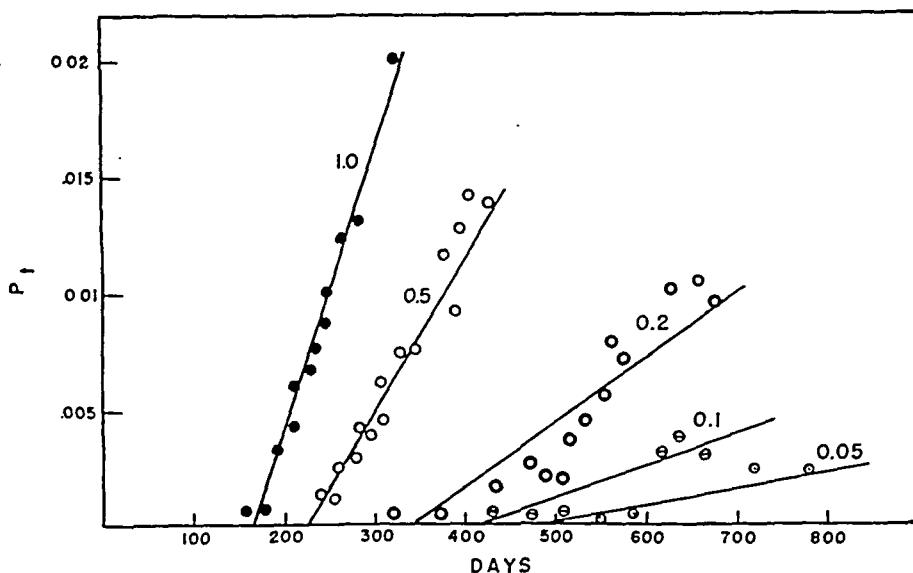


FIG. 2. DAILY PROBABILITY OF BONE TUMOR DEVELOPMENT (P_t) AS A FUNCTION OF TIME AFTER BEGINNING OF MONTHLY INJECTIONS

Figures beside the curves indicate dosages in $\mu\text{c/g}$.

may develop than in experimental animals. One semiquantitative expression of this has been stated (14). The data shown in Figure 2 may be interpreted in this way: that each quantity of absorbed radiation confers on the tissue absorbing it a probability of tumor formation which is *without limit in time*, once the latent period is passed; thus the daily tumor morbidity will constantly increase as long as further irradiation occurs.

I am indebted to Dr. R. D. Evans (31) for pointing out that the data shown here are also consistent with a Gaussian distribution of tumors around an optimal time after treatment, where the parameters are specified functions of dose. It would be virtually impossible to distinguish between these two expressions with such experimental data as can be obtained by the techniques used here.

When a single injection of Sr^{89} is given, we

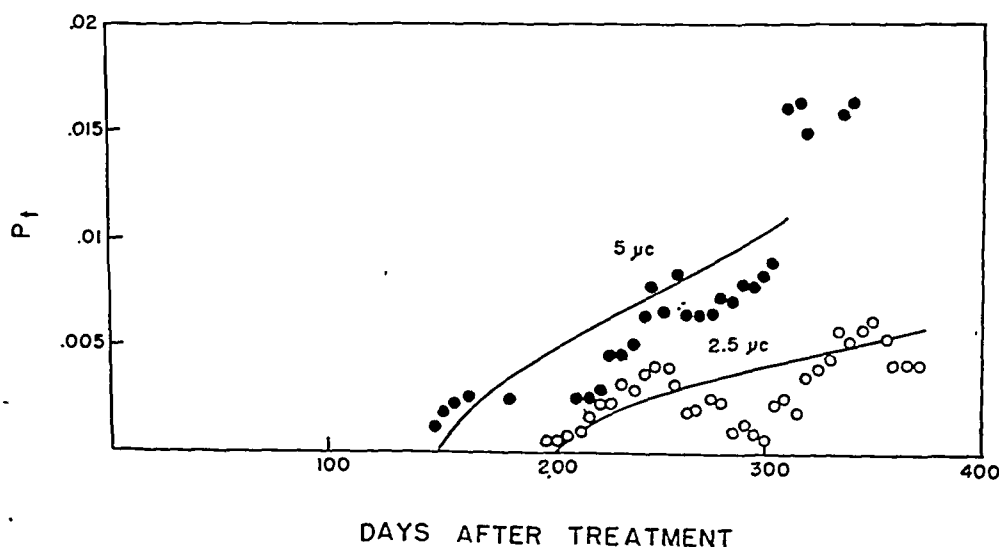


FIG. 3. DAILY PROBABILITY OF TUMOR DEVELOPMENT AS A FUNCTION OF TIME AFTER A SINGLE INJECTION OF Sr^{89} , AS IN FIGURE 2

might expect to see a somewhat simpler function. That this is not the case appears from Figure 3. Here we again see a sharply defined latent period, followed by an increase in tumor morbidity. These data also indicate that the subsequent morbidity is, within the limitations imposed by survival, relatively without limit in time after the latent period is passed.

Further data regarding the pattern of tumor formation in mice and in other species will be published in the future, when the pathologic data are complete. The preliminary results suggest that (1) in rats and mice, the tumor incidence following radium administration gives a ratio of effectiveness of radium: strontium⁸⁹ of about 10:1 on a millicurie basis; and (2) that the response to bone-seeking radioelements as regards latent period and tumor incidence per animal is comparable as between rats, mice, and rabbits, and is certainly no greater in dogs.

DISCUSSION

Such data as have been obtained from a variety of sources still fail to answer a number of questions which must be understood before it will be possible to make direct application of experimental data to man, especially in the tolerance range.

In the first place, the question whether there is a true threshold for carcinogenic effects appears to be entirely unanswered. The scantiness of human data on the one hand, and the short life-span of animals on the other, makes it impossible to say what effects might be seen in a large human population after a long period of time. The human experience has been reviewed by Evans (32) in some detail, and the author concludes that total dosages of 1000 to 10,000 rep for alpha radiation have been required to induce an incidence of 10 per cent tumors after 15 years. The X-ray data previously cited (11) and the bulk of animal data involving absorbed isotopes leave the impression that the lowest amount of X-radiation or beta radiation which has been clearly shown responsible for carcinogenesis (excepting mouse lymphoma) may be of the same order. It cannot be said whether underlying visible tissue changes are necessary to radiation carcinogenesis, or whether it is a statistical event that may (with a very low probability) occur whenever a cell lies in the path of ionization.

Secondly, the relation between life-span of a

given species and the latent period before tumor formation requires study. In the matter of chemical carcinogenesis, it appears that the period of latency for the dog and man is longer than in the rodents. We are not aware that such a relationship has clearly been shown for radiation-induced neoplasia under controlled conditions.

Finally, the concept of the origin of malignant disease (whether a stray statistical event taking place in a single cell, or a systemic change) has considerable bearing on the relation between human and experimental results. The former concept is consistent with the observed distribution of tumors within a group of animals, since multiple primary bone tumors do not appear to occur with undue frequency. Assuming that cancer is a disease arising in a single cell, and that all the cells of a tissue are more or less equally susceptible to the change, it can be seen that even under a strong stimulus the carcinogenic event has a numerical rarity comparable to the incidence of genetic mutations. In that event, man might be expected by virtue of mass and cell number to have a susceptibility to tumor formation on the order of 1,000 times that of the mouse. It will be obvious that this is not the case, and that if it were, the survival of the larger species would be impossible. Thus the extrapolation of the carcinogenic effects of radiation from rodents to man involves unknown variables in several parameters, and poses some of the basic problems in cancer biology.

SUMMARY

The effects of large dosages of radioisotopes resemble to a marked extent those of total-body radiation, with modifications dependent on tissue distribution. Permissible exposure levels are determined by the late or chronic radiation changes, of which malignant changes have quite properly attracted major attention.

There is no evidence to indicate that radiation dosages to tissue corresponding to established "permissible" external dosages (0.3 — 0.6 rep/week) will result in an increased incidence of malignant disease detectable in a population of moderate size. In making such calculations, it is necessary to estimate maximum local tissue dosage, rather than the mean value for an organ or structure, and to make use of factors recognizing the greater effectiveness of ionization in areas

where it is dense (*e.g.*, alpha-particle ionization).

The suggestion is made that a more conservative figure is desirable where a large population may be affected, and the basis for using larger dosages in clinical investigation, diagnosis, and therapy is discussed.

Bone tumor induction by Sr^{90} has been studied extensively in mice. The data are consistent with the view that each given quantity of radiation to bone confers a given probability of bone tumor formation which may occur at any time following a "latent period." The latent period increases gradually with the dosage. It is not known at present whether a threshold exists for such effects.

ACKNOWLEDGMENT

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EQUATIONS FOR TRACER EXPERIMENTS

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The use of isotopes makes possible quantitative measurements of many biochemical and physiological processes, hitherto secure from such detailed observation. Perhaps the most frequent use of this tool is in the observations of physiology that are concerned with the transfer of fluid from one portion of the body to another, and in the biochemical observations of the formation of one compound from another. It is the purpose of this paper to present in detail some of the mathematical equations that govern such flow and synthesis, and to show incidentally that many of these equations are common to the fields of physics, and chemistry, as well as to the biological disciplines to which they will be applied here. A series of case studies will be presented, including, as examples, the formation of cholesterol from deuterium-marked body water, the uptake of phosphorus by the red blood cell and the flow of sodium out of the plasma into cerebrospinal fluid.

Mathematical analysis of this kind is certainly not novel; indeed, Burton (1) reports that Harcourt and Esson (2) concerned themselves with similar studies in 1866 when chemical kinetics was just beginning. In another connection, Rutherford, Chadwick and Ellis (3) have expressed some equations governing the familial relationship between radioactive parent elements and their daughters; they report that Bateman (4) as early as 1910 developed a theory which covers the general case of radioactive decay and recovery. The mathematics apply so generally that any survey of the literature must necessarily be incomplete.

1. Basic assumptions

The basic assumption that underlies all the equations to be developed is that the tracer element follows its unlabeled isotope faithfully in all biological reactions. Qualitatively there is good agreement on this point; quantitatively, evidence is gradually accruing that the rates of reaction are influenced by the isotopic composition of the reacting molecules. However, no

allowance has been made for this in the cases to be discussed. Implicit in this primary assumption is the condition that the injection of the tracer element shall not disturb, in any important fashion, the normal metabolic behavior of the system. As a consequence of this initial assumption, the equations to be derived apply to unlabeled systems also; the tracer provides the method of measurement.

The second basic assumption is that in systems of constant volume the rate of flow of isotope out of a compartment is proportional to the amount of labeled isotope present in the compartment. This reduces to the assumption that rate is proportional to *concentration*, given constant volume. The difference is purely formal and can be taken up by suitable adjustments of the constants involved. Thus, the investigator has his choice of expressing the activity as, say, counts per milliliter, or counts per milliliter times the volume of the compartment in milliliters. The equations that follow have been based on amount. In systems in which the volume is not constant, other assumptions are made.

A third basic assumption is that of uniform distribution throughout the compartments. There are many physiological conditions in which this assumption is invalid, as when the time of mixing is long compared to the reaction rate, or when the viscosity of the fluid in the compartment is high. In many cases, the experimental conditions can be adjusted in order to make these factors unimportant; if not, suitable corrections must be applied.

2. Symbols

P = total amount of labeled material in compartment A.

P_0 = P at time 0.

t = time.

k_{ab} = coefficient of transfer from compartment A to compartment B.

p = concentration of labeled material in compartment A.

- v_p = volume of compartment A.
 Q = total amount of labeled material in compartment B.
 k_{ba} = coefficient of transfer from compartment B to compartment A.
 q = concentration of labeled material in compartment B.
 v_a = volume of compartment B.
 k_{ao} = coefficient of transfer from compartment A to outside.
 etc.

3. Case 1—Simple decay

The decay of a radioactive isotope can be described by an equation which states that the rate of loss of isotope depends upon the amount of isotope remaining. This is perhaps the most common example of simple decay. In chemistry, monomolecular reactions are governed by a similar equation, since the rate of reaction is dependent on the amount of reactant remaining (though not necessarily independent of concentration). Margenau and Murphy (5) point out that the rate of growth of bacterial cultures in an unlimited nutrient medium is dependent on the number of bacteria present; thus the equation is similar to that for radioactive decay, though the sign, of course, is different.

$$1-1 \quad \frac{dP}{dt} = -k_{so}P$$

$$1-2 \quad P = P_0 e^{-k_{so}t}$$

$$1-3 \quad \ln P = \ln P_0 - k_{so}t$$

$$1-4 \quad t_{\frac{1}{2}} = 0.693/k_{so}$$

Figure 1 shows the decay of radioactive phosphorus (P^{32}). The amount of this material remaining, P (expressed as relative counts/minute in a unit mass of sample) is plotted on a logarithmic scale against t . The slope of this straight line gives k_{so} , from which the half-life, $t_{\frac{1}{2}}$, may be derived by equation 1-4. In this case, $t_{\frac{1}{2}}$ measures the time required for half the material to disintegrate.

4. Case 2—Uptake from a constant source

This case arises most frequently in stable isotope experiments in which the body fluids are brought to a constant deuterium level, and the uptake of deuterium in various compounds is

examined. The basic assumption is that the isotopic content of the circulating fluids remains constant. This includes the implicit assumption that the return of material to the circulating fluids from the compartment examined is unimportant.

This example of Case 2 is shown diagrammatically in Figure 2. Here compartment A represents the body fluids, and P represents the total amount of deuterium in the fluids. The concentration, p , is given by the atom per cent deuterium in the body fluids. Compartment B represents the cholesterol; Q is the total amount of deuterium in the cholesterol, and q is the concentration of deuterium given by atom per cent deuterium in the cholesterol (usually referred to atom per cent D in the body fluid as 100%).

Another common example of this case is the formation of radioactive isotopes in nuclear reactors. The basic assumption in this case may be restated: the flux of neutrons must remain constant, and be unaffected by the products formed in the material being irradiated.

$$2-1 \quad \frac{dQ}{dt} = k_{ab}P - k_{bo}Q, \quad P = P_0 = \text{const.}$$

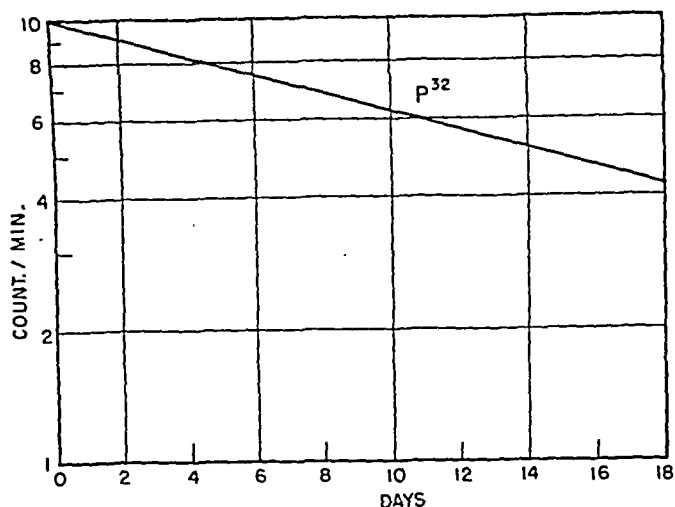
$$2-2 \quad Q = \frac{k_{ab}}{k_{bo}} P_0 (1 - e^{-k_{bo}t})$$

$$2-3 \quad Q_{\infty} = \frac{k_{ab}}{k_{bo}} P_0, \quad t = \infty$$

$$2-4 \quad \ln(1 - Q/Q_{\infty}) = -k_{bo}t$$

$$2-5 \quad t_{\frac{1}{2}} = 0.693/k_{bo}$$

These equations differ materially from those of simple decay. For one thing the half-life is dependent upon the rate of decay of the material in compartment B, not on the rate of its synthesis, unless an equilibrium obtains, in which case the two rates are equal. The dependence of half-life upon decay may be illustrated by considering the formation of isotopes in reactors. For a given rate of decay, that is, a given half-life, the *amount* of material formed depends directly on the intensity of the neutron flux. The *time* it takes to achieve the maximum formation of radioactive material is dependent on the half-life and independent of the neutron flux. That is, more radioactive material can be formed by an increase in neutron flux, but the time it takes to reach the new maximum value is independent of this increase in neutron flux.

FIG. 1. CASE 1: RADIOACTIVE DECAY OF P^{32}

Relative counts/min. in a unit mass of sample is plotted on a logarithmic scale against time.

In order to determine whether the equation 2-1 holds for conditions in which the volume is not constant, the equation 2-1 has been fitted to experimental results on the uptake of deuterium by maternal and fetal cholesterol reported by Goldwater and Stetten (6). Pregnant rats put on a heavy water regimen in the 18th day of term were sacrificed at quarter-day intervals from the 18th to the 19th day, and at half-day intervals from the 19th to the 20th day. One control was kept on the heavy water regimen for the total 20-day term in order to determine the maximum uptake of deuterium in the fetal cho-

lesterol, and the maternal cholesterol, both liver and carcass. In the two-day period between the 18th and the 20th day, the weight per fetus varied between 2 and 5.2 grams. The tissue content of cholesterol in all three cases showed a slight increase. Thus, the amount of cholesterol, the volume as it were, of compartment B is certainly not constant.

The weight increase in the maternal rat will show whether constant isotope concentration in the body fluids is equivalent to a constant amount of deuterium in the fluids. If one ascribes the weight increase to increase in fetal

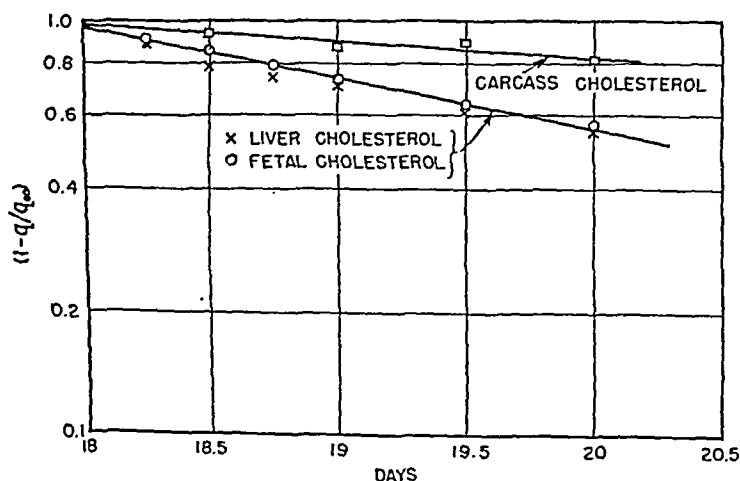
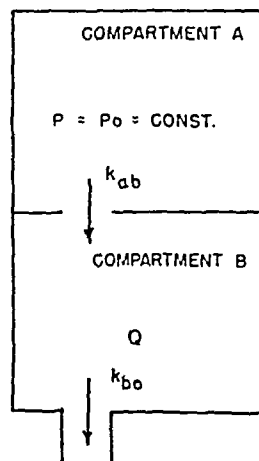
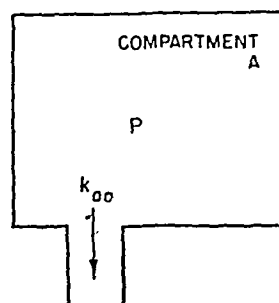


FIG. 2. CASE 2: UPTAKE OF DEUTERIUM BY CHOLESTEROL IN THE MATERNAL RAT AND FETUS

The function $(1 - q/q_\infty)$ is plotted on a log scale against time. The slope of the curve determines the transfer coefficient k_{bo} .



weight alone, Goldwater and Stetten's figures show that the average increase in maternal rat weight in the two-day period is about 11%. This is small compared to the other changes in the system; hence, we will assume that both concentration and amount of deuterium in the body fluids remain constant.

If the assumptions in equation 2-1 are correct, the function derived in equation 2-4 should fall on a straight line when plotted against time. Since it does not, a different assumption must be made. We may test the assumption that the rate of change of the *amount* of labeled cholesterol is proportional to the *concentration* of deuterium in the body fluids, and the *concentration* of deuterium in the cholesterol. Stated analytically, this becomes:

$$2-6 \quad \frac{dQ}{dt} = k_{ab}P - k_{ba}Q$$

If one assumes that the change in cholesterol content in the fetus varies linearly with time, an assumption supported by the data, this equation can be solved. In this case, too, the equation is seen to fail in describing the data. However, when one assumes that the rate of change of *concentration* of cholesterol is proportional to the *concentration* of deuterium in the body fluids and the *concentration* of deuterium in the cholesterol the equation fits the data. In this case the equation becomes

$$2-7 \quad \frac{dq}{dt} = k_{ab}P - k_{ba}Q$$

and 2-4 reduces to

$$2-8 \quad \ln(1 - q/q_{\infty}) = -k_{ba}t$$

The calculations are given in Table I, and the function is plotted in Figure 2. It will be seen that all three cases fit the equation nicely, even though the volume in compartment B is greatly changed during the course of the experiment. Many chemical systems can be postulated that can be described analytically by equation 2-7.

5. Case 3—Closed two-compartment system

The equilibration of red blood cells with K^{42} -marked plasma is used as an example of this case. The basic assumption is that the total amount of labeled material in the system remains constant. In Figure 3 this case is shown diagrammatically. Compartment A represents the

plasma containing an amount P of K^{42} , and compartment B represents the erythrocytes containing an amount Q of K^{42} .

Since Cohn and Cohn (7) investigated the permeability of the red corpuscles of the dog to sodium ions, many experiments have been carried out on cellular permeability. Sheppard (8) has recently reviewed these experiments and reported some additional results of his own. The equations he derives, specific examples from a previously published general treatment (9), correspond with those to be derived below.

$$3-1 \quad P + Q = P_0 = \text{const.}$$

$$3-2 \quad \frac{dP}{dt} = -k_{ab}P + k_{ba}Q$$

$$3-3 \quad \frac{dQ}{dt} = k_{ab}P - k_{ba}Q$$

$$3-4 \quad Q = \frac{k_{ab}}{k_{ab} + k_{ba}} P_0 [1 - e^{-(k_{ab} + k_{ba})t}]$$

$$3-5 \quad Q_{\text{equil}} = \frac{k_{ab}}{k_{ba}} P_{\text{equil}}$$

$$3-6 \quad Q_{\infty} = \frac{k_{ab}P_0}{k_{ab} + k_{ba}}$$

$$3-7 \quad \ln(1 - Q/Q_{\infty}) = -(k_{ab} + k_{ba})t$$

$$3-8 \quad t_{10} = 0.693/(k_{ab} + k_{ba})$$

$$3-9 \quad P = \frac{k_{ba}P_0}{k_{ab} + k_{ba}} \left[1 + \frac{k_{ab}}{k_{ba}} e^{-(k_{ab} + k_{ba})t} \right]$$

$$3-10 \quad P_{\infty} = \frac{k_{ba}P_0}{k_{ab} + k_{ba}}$$

$$3-11 \quad \ln(P/P_{\infty} - 1) = -(k_{ab} + k_{ba})t - \ln \frac{k_{ba}}{k_{ab}}$$

The example chosen to illustrate this case has been taken from some unpublished work of Raker, Taylor and Weller (10) on the uptake of K^{42} by human erythrocytes in a dilute solution of K^{42} -marked plasma. Figure 3 shows the function $(P/P_{\infty} - 1)$ plotted on a logarithmic scale against time. The constants describing the system may be determined as shown in equation 3-11 from the slope and intercept of this line. The calculations have taken account of a slight swelling of the cells towards the end of the experiment, as shown by a 10% increase in the hematocrit. This change in volume of compartment B is so small, however, that it does not affect the fit of the equation. The function $(P/P_{\infty} - 1)$ in equation 3-11 is plotted on a logarithmic scale against time in Figure 3. A curve drawn on the assumption that the flow of

TABLE I

Uptake of deuterium in pregnant rats whose body fluids were enriched with D₂O
 Calculations from data presented by Goldwater and Stetten to obtain the function $(1 - q/q_{\infty})$.

Days on D ₂ O	Fetal cholesterol			Liver cholesterol			Carcass cholesterol		
	D	$q/q_{\infty} = D/D_{\infty}$ *	$1 - q/q_{\infty}$	D	$q/q_{\infty} = D/D_{\infty}$	$1 - q/q_{\infty}$	D	$q/q_{\infty} = D/D_{\infty}$	$1 - q/q_{\infty}$
	<i>per cent body water</i>			<i>per cent body water</i>			<i>per cent body water</i>		
18-18½	5.0	0.090	0.910	4.8	0.105	0.895			
18-18½	8.2	0.148	0.852	10.5	0.229	0.771	2.5	0.072	0.928
18-18¾	11.3	0.204	0.796	11.3	0.246	0.754			
18-19	15.4	0.279	0.721	13.5	0.294	0.706	4.7	0.135	0.865
18-19½	20.4	0.368	0.632	17.7	0.386	0.614	3.9	0.112	0.888
18-20	24.2	0.438	0.562	19.8	0.432	0.568	6.7	0.193	0.807
1-20	55.4	—	—	45.8	—	—	34.8	—	—

* D₂₀ represents the D of the various fractions for the rat which was fed D₂O for the total 20-day period.

K⁴² is determined by its *concentration* in plasma and cells fits the data equally well, and is probably preferable on theoretical grounds.

The symmetry of this system is very interesting. The observations of the time course can be made on either plasma or cells, provided that

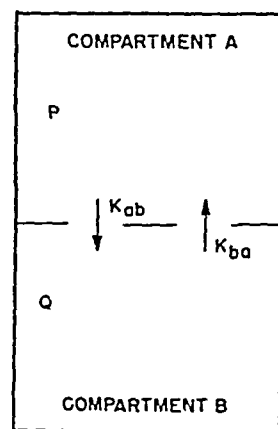
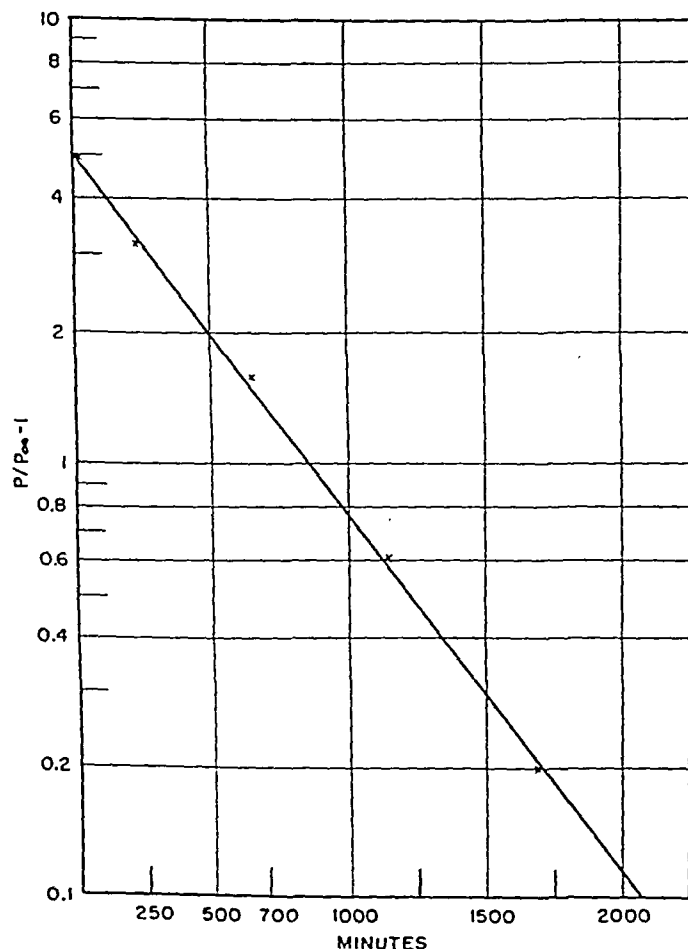


FIG. 3. CASE 3: UPTAKE OF K⁴² BY RED BLOOD CELLS FROM PLASMA

The function $P/P_{\infty} - 1$ is plotted on a logarithmic scale against time. From the slope and intercept of this curve the transfer coefficients k_{ba} and k_{ab} may be determined.

the equilibrium amounts in both compartments be determined. Indeed, it is theoretically possible, though not experimentally desirable, to characterize this system exactly by only three measurements: the amount of isotope in either component at any time t , prior to equilibrium, and the equilibrium amounts.

6. Case 4—Diffusion or flow out of a compartment

An example of flow out of a compartment is the escape of sodium from the blood stream following a single intravenous injection. Merrell, Gellhorn and Flexner (11) have derived equations which describe this process in the guinea pig. In the case of the guinea pig, the process can be described by a simple exponential similar to, but not identical with, that given in Case 3, in which compartment A is the plasma and P is the amount of tracer sodium in the plasma. Merrell *et al.* assume that the process of sodium movement is one of diffusion, that is, that the coefficient of transfer into the plasma equals the coefficient of transfer out when the equation is expressed in terms of concentration of sodium in the various compartments. Apparently, it is assumed implicitly that there is no sodium excretion during the course of the experiments.

In the dog (Gellhorn, Merrell, and Rankin [12]) and in man (Flexner, Cowie, and Vosburgh [13] and Burch, Reaser, and Cronvich [14]), the process is described by at least two separate rates. A two-rate system (neglecting return from extra-cellular fluid to plasma) implies the presence of a split compartment in the extra-cellular fluid, rather than a compartment with two leaks of different size. Two leaks of different size are equal to one leak of larger size, and can be described by a single transfer coefficient. However, as Gellhorn *et al.* point out (12), the situation is far different when the return of Na^{24} to the plasma is taken into account. Assume that the Na^{24} escapes from the plasma into two separate external compartments as shown diagrammatically in Figure 4, entering one quickly and the other slowly. The return from these two compartments to the plasma must be characterized by two different rates, since the amount of tracer in compartment B is different from the amount in compartment C.

Thus, the flow of Na^{24} from the plasma will be described by an equation containing at least two exponentials. It is not assumed that the compartments B and C exist as such in the body, but rather that one, say B, represents the average of a number of areas to which the sodium is transferred quickly, and the other, C, the average of a number of areas to which the sodium is transferred more slowly.

In the treatment that follows, three basic assumptions will be made: first, that the volume of the compartments remains constant; and second, that there is no excretion of Na^{24} during the period of observation. For simplification, we will also assume that the coefficient of transfer across the membrane separating the compartments is the same in both directions. That is,

$$4-1 \quad k_{ab} = k_{ba} = k_1; \quad k_{ac} = k_{ca} = k_2$$

The equations that follow are similar to those derived by Gellhorn *et al.*, and the reader is referred to Appendix 1 of reference 12 for the derivation.

$$4-2 \quad \frac{dP}{dt} = -k_1P + k_1Q - k_2P + k_2R$$

$$4-3 \quad \frac{dQ}{dt} = k_1P - k_1Q$$

$$4-4 \quad \frac{dR}{dt} = k_2P - k_2R$$

$$4-5 \quad P + Q + R = P_0$$

$$4-6 \quad \frac{d^2P}{dt^2} = -2(k_1 + k_2) \frac{dP}{dt} - 3k_2k_1P + k_2k_1P_0$$

$$4-7 \quad P = a_1e^{-b_1t} + a_2e^{-b_2t} + \frac{1}{3}P_0$$

where

$$a_1 = \frac{1}{3}P_0 + \frac{1}{6}P_0 \frac{(k_1 + k_2)}{\sqrt{k_1^2 - k_1k_2 + k_2^2}}$$

$$a_2 = \frac{1}{3}P_0 - \frac{1}{6}P_0 \frac{(k_1 + k_2)}{\sqrt{k_1^2 - k_1k_2 + k_2^2}}$$

$$b_1 = (k_1 + k_2) + \sqrt{k_1^2 - k_1k_2 + k_2^2}$$

$$b_2 = (k_1 + k_2) - \sqrt{k_1^2 - k_1k_2 + k_2^2}$$

$$4-8 \quad \text{At equilibrium,}$$

$$P = Q = R = \frac{1}{3}P_0$$

In the course of studies now in progress to determine the uptake of Na^{24} by cerebrospinal fluid, Sweet, Solomon, and Selverstone (15) have examined the diffusion of this isotope from human plasma. The results have been plotted in Figure 4, and analyzed on the basis of the equation 4-7. For the purpose of this example, a smooth curve drawn through the experimental

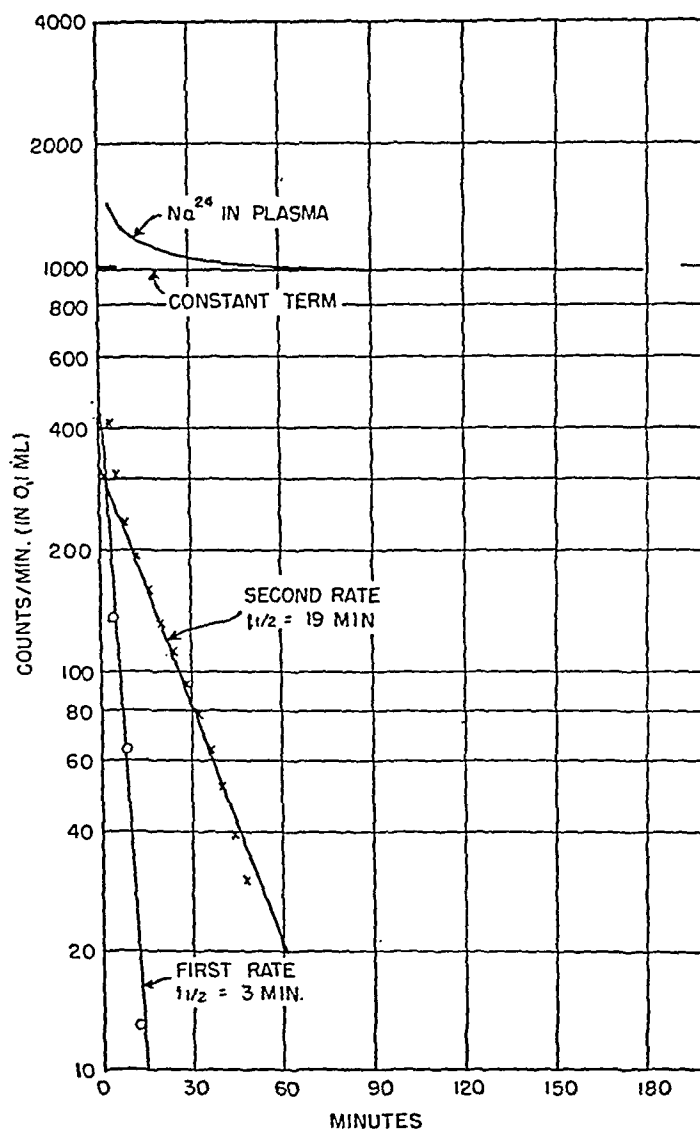


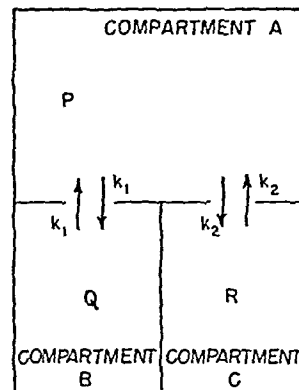
FIG. 4. CASE 4: DISAPPEARANCE OF Na^{24} FROM PLASMA IN MAN

Counts/min. in 0.1 ml in the plasma is plotted on a logarithmic scale against time. The transfer coefficients may be determined from the slopes and intercepts of the first and second rates, and the value of the constant term.

points has been used; the full data will be published elsewhere. In Figure 4, the radioactivity has been expressed as counts per minute in 0.1 ml, plotted on a logarithmic scale against time; since constant volume is assumed, this expression is a valid index of the total amount of radioactivity, P. In order to analyze a curve plotted in this way into its components, the constant term is first subtracted graphically from the curve. The difference between these two curves, shown in Figure 4 as the crosses, is then plotted, and the straight line is drawn which best fits these points. That straight line gives

the second rate and has the slope $-b_2$. It, in turn, is subtracted from the points represented by the crosses that do not fall on the line at the beginning of the period of measurement. Thus, the first rate, given by the circles on Figure 4, is obtained. The method of analyzing experimental curves by a sum of exponentials with constant coefficients is so powerful, and the number of adjustable constants so large, that great care must be taken in interpreting such curves physiologically.

Accepting this curve as a formal description of the behavior of the labeled material in the



plasma, one would expect that a knowledge of the total amount of injected material, and the intercept of the curve of concentration of Na^{24} at zero time, would lead to a good value of the total plasma volume. In the dog, Gellhorn *et al.* (12) were unable to obtain reliable results by the use of this method, a conclusion supported by the preliminary experiments in man of Sweet, Solomon, and Selverstone (15). This may be due to incomplete knowledge about the early part of the curve.

7. Case 5—Uptake of radioactive material in a three-compartment system

An example of this case is found in the uptake of Na^{24} by ventricular cerebrospinal fluid, follow-

ing intravenous injection. In order to determine the coefficient of transfer into the cerebrospinal fluid, it is not necessary to accept any hypothesis as to the physiological basis for the time curve of isotope in the plasma; it suffices to express the curve analytically. The equations that follow have been developed on the basis of the plasma equation given in Case 4, as equation 4-7. As can be seen from the diagram in Figure 5, the conditions are not exactly identical with those assumed for Case 4. Then we assumed that the coefficient of transfer from compartment A to compartment B was equal to that of transfer in the reverse direction. As has been shown, an equation can be derived on those assumptions, which describes the time course of the tracer

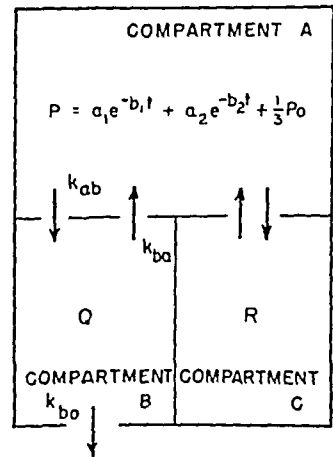
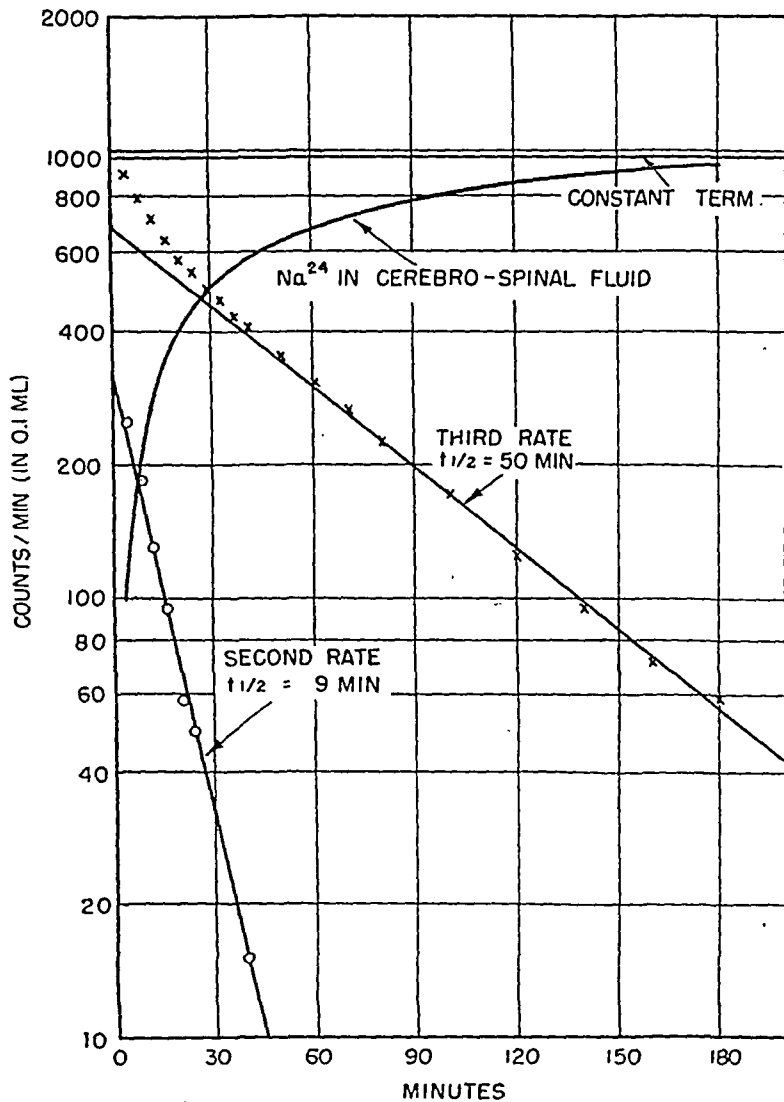


FIG. 5. CASE 5: UPTAKE OF Na^{24} BY CEREBROSPINAL FLUID FROM THE PLASMA

Counts/min. in 0.1 ml in the cerebrospinal fluid is plotted on a logarithmic scale against time. The transfer coefficient k_{ab} and the sum of coefficients ($k_{ba} + k_{bc}$) can be determined from the slopes and intercepts of the second and third rate and the value of the constant term.

content of the plasma. In the case of cerebrospinal fluid, we will assume that the volume of the compartments and their Na concentration is constant.

We will represent the plasma as before by compartment A, and the ventricle as compartment B. The coefficient of transfer k_{bo} will represent the flow of Na^{24} out of the ventricle toward the cistern. Since some of the Na^{24} will find its way back to the plasma by this route, k_{bo} and k_{ba} could, if desired, be represented together as a single constant. The equations to be derived in this case do not permit their individual determination.

$$4-7 \quad P = a_1 e^{-b_1 t} + a_2 e^{-b_2 t} + \frac{1}{3} P_0$$

$$5-1 \quad \frac{dQ}{dt} = k_{ab}P - k_{ba}Q - k_{bo}Q = k_{ab}P - (k_{ba} + k_{bo})Q$$

$$5-2^* \quad Q = \frac{k_{aba_1}}{(k_{ba} + k_{bo}) - b_1} e^{-b_1 t} + \frac{k_{aba_2}}{(k_{ba} + k_{bo}) - b_2} e^{-b_2 t} + (k_{ba} + k_{bo})e^{-(k_{ba}+k_{bo})t} + \frac{k_{ab}}{3(k_{ba} + k_{bo})} P_0$$

5-3 At equilibrium

$$P = \frac{(k_{ba} + k_{bo})Q}{k_{ab}}$$

Figure 5 presents a smoothed curve drawn from the experimental results of Sweet, Solomon, and Selverstone (15). The same method has been applied in plotting and analyzing this curve as has been used with Figure 4. The values of the transfer coefficients can be obtained from the slopes and intercepts of the various segments of the curve, since a_1 , a_2 , b_1 , b_2 are all known, at least empirically, from the curve presented in Figure 4. It will be noted that the analysis in Figure 5 does not include the first rate of Figure 4 with its half-life of three minutes. A further test of the validity of equation 5-2 is available by comparing the slopes of the second term of equation 4-7 ($t_{\frac{1}{2}} = 19$ mins.) with the same term ($t_{\frac{1}{2}} = 9$ mins.) in 5-2. It is seen that the agreement is not good. It would perhaps be surprising to obtain good agreement under these conditions, since no account has been taken of the viscosity of the cerebrospinal fluid, nor has any allowance been made for mixing time. In so complex a system, it must be emphasized that the equations give only a formal description. Many experiments must be carried out in

order to determine whether such a mathematical description fits the physiological facts.

8. Ultra-filtration vs. secretion

Kinsey and Grant (16) in their studies on the uptake of tracers by the aqueous humor have pointed out that the equilibrium values for the components in tracer systems are not in themselves sufficient to decide what mechanism is operating. Consider Case 2 and equation 2-7, as an example of secretion

$$2-7 \quad \frac{dq}{dt} = k_{ab}p - k_{bo}q$$

Here p and q are the concentrations in compartments A and B, and return from compartment B to compartment A is excluded.

If it is assumed, as in ultra-filtration, that the barrier between compartments A and B is a simple membrane, and that the coefficient for transfer across that membrane (with respect to concentration) is the same in both directions, equation 2-7 becomes

$$6-1 \quad \frac{dq}{dt} = k_{ab}p - k_{ab}q - k_{bo}q$$

The solution for 2-7 (secretion) is

$$6-2 \quad q = \frac{k_{ab}}{k_{bo}} p_0 (1 - e^{-k_{bo}t})$$

and for 6-1 (ultra-filtration)

$$6-3 \quad q = \frac{k_{ab}}{k_{ab} + k_{bo}} p_0 (1 - e^{-(k_{ab}+k_{bo})t})$$

Since $k_{ab} + k_{bo}$ can be represented by another constant, say k_{bb} , it is not, in general, possible to decide between these two hypotheses without additional independent measurements of one of the constants. If, however, the constant in the denominator of equation 6-3 is less than k_{ab} , it is apparent that the mechanism is not one of ultra-filtration.

Another example of this problem is afforded by the uptake of Na^{24} by cerebrospinal fluid. As has been pointed out, the sum of the constants $k_{ba} + k_{bo}$ in equation 5-2 can easily be determined, but the individual values cannot be obtained independently without further measurement.

9. Precursors

Zilversmit, Entenman, and Fishler (17) have put forward a criterion that states that one compound, A, is the precursor of a second, B, if, when the specific activity of B has reached its maximum, it equals the specific activity of A. Let us examine this criterion on the basis of our

* For details of the solutions of such differential equations, see the relevant chapter in reference (5).

Case 2 which is similar to that described by Zilversmit *et al.*

Let the specific activity in A be denoted by $P_s = P/P^*$ where P^* is the total amount of unlabeled isotope in compartment A, and similarly, let $Q_s = Q/Q^*$.

From 2-1

$$\frac{dQ}{dt} = k_{ab}P - k_{ba}Q$$

for $Q = \text{maximum}$

$$10-1 \quad \frac{dQ}{dt} = 0 = k_{ab}P_s P^* - k_{ba}Q_s Q^*$$

$$10-2 \quad k_{ab}P_s P^* = k_{ba}Q_s Q^*$$

Since $P_s = Q_s$ to satisfy the criterion

$$10-3 \quad k_{ab}P^* = k_{ba}Q^*$$

Equation 10-3 states that the amount of unlabeled isotope in compartment B is in equilibrium, in agreement with the assumptions initially made by Zilversmit *et al.*

Another criterion may be found by consideration of a chain of reactions. Let $J \rightarrow K \rightarrow L$, in a series of unimolecular reactions which can be described by the basic assumptions in section 1. The rate of growth of K from J will be given by an equation with a single exponential term, as shown in Cases 2 and 3. The rate of growth of L from J, however, will be governed by an equation which requires two exponential terms to describe it, and each succeeding member in the chain, M, N, and O, will require an additional exponential term in the equation (Burton [1]). Thus, we can say, if J is the precursor of K, a single exponential will suffice to describe the growth of K from J. If our basic assumptions describe the reaction correctly, the addition of a second exponential in the expression for growth must mean a two-step process.

10. Discussion of mathematical methods

As has already been mentioned, the use of differential equations to describe steady states of this kind is not new. Indeed, Burton (1) derived his description of the steady state in 1936 with no reference to isotopes. The most general treatment of this kind is that given by Sheppard (9). Tobias (18) has derived a system of equations describing the movement of isotopes under conditions of constant volume. Burch, Threefoot, and Cronvich (19) have examined the effect of changes both of compart-

ment volume and amount of unlabeled isotope on the constants involved in such equations.

It is also possible to describe the behavior of tracer systems by the use of integral equations. Shemin and Rittenberg (20) have made use of an integral equation in order to determine the life span of the human red blood cell using glycine-labeled hemin, a case very different from those considered here, since the decrease of labeled activity is a function of the age of the red blood cell in which it is contained. Branson (21) has also used integral equations in order to describe isotope systems.

SUMMARY

Equations have been developed and discussed covering five common cases of the use of isotopes in biological and physiological systems.

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THE THERAPEUTIC APPLICATION OF RADIOACTIVE PHOSPHORUS WITH SPECIAL REFERENCE TO THE TREATMENT OF PRIMARY POLYCYTHEMIA AND CHRONIC MYELOID LEUKEMIA¹

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The use of radioactive phosphorus in the treatment of disease stems from the initial studies made by Lawrence and his colleagues in 1936 (1). Since then there have been a number of reports which establish the efficacy of this form of therapy in chronic leukemia and polycythemia (2-11). These clinical reports have been issued from a number of separate institutions, indicating that independent experiences are now fairly widespread. The report by Reinhard *et al.* (12) is an exhaustive review of the clinical course of patients treated in this fashion. Similar studies by Doan *et al.* (13) and Lawrence *et al.* (14) add extensively to the data now available in the literature. It is our intention here to add to the clinical observations presented in the previous reports, but we wish particularly to emphasize the pertinent information available from the literature and from our own work which is the basis and rationale for such treatment.

The concept of treating disease with internally distributed radioactive materials is not completely new and attempts in this direction have previously been made by introducing into the blood stream some of the naturally occurring elements, such as radium. However, the invention of the cyclotron and more recently the development of high energy nuclear reactors of the Atomic Energy Commission have given considerable impetus to this concept of therapy. This is particularly true because of the wide variety of radioactive elements which can be produced by these devices, some of which have suitable characteristics (desirable half-life, proper radiation characteristics, and satisfactory chemical properties).

The rationale of treating patients with radioactive material arises from the desire to irradiate selectively certain specific tissues and to prolong

the irradiation over one or more life cycles of the cells to be irradiated. Ever since it became clear that ionizing radiation could affect malignant tumors and allied conditions in a beneficial manner, selective localization has been continually sought after. The radiation would thus be localized primarily in a specific tissue or organ, sparing the normal remaining tissue from any deleterious effect. Even when external radiation is employed, an effort is made to localize the radiation in a specific site. This is achieved with external X-rays by means of cross-firing techniques and sharply limited portals.

At the present time the outstanding example of selective localization concerns itself with the deposition of iodine in the thyroid gland and the use of radioactive iodine for treatment of diseases of this gland arises from this highly specific localization.

In the case of P^{32} , a similar though much less pronounced selective deposition exists in leukemic tissue, in the erythrocytogenic tissues in primary polycythemia, and in certain rapidly growing tumors. Moreover, P^{32} is preferentially deposited in bone, since phosphate is a continuous requirement and important constituent of bone. This further adds selective radiation to the bone marrow which, in general, is the important site of the two diseases noted above.

In addition to selective localization in the case of various radioactive elements, the differential sensitivity of cells during various phases of the mitotic cycle must be considered. If it is true that cells are affected by radiation only during certain phases of the mitotic cycle, then prolonged low-grade radiation such as that which can be achieved by the use of P^{32} may be highly desirable. The fact that rapidly metabolizing, poorly differentiated tissues are sensitive to radiation may be another important factor.

It is known that phosphorus introduced into the blood stream in the form of a readily diffusible

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and ionizable phosphate will be available to all tissues of the body and those tissues which utilize phosphorus in their metabolic processes will rapidly take up this element. The rate of uptake and the deposition of phosphorus—and here there is no distinction between radioactive phosphorus and ordinary phosphorus—is a function of several things.

1. *The metabolic turn-over rate* will determine the rate at which a specific cellular structure will reach equilibrium with its milieu. This is dependent upon (a) the metabolic activity of the tissue, and (b) its rate of growth. The two may be closely intertwined and inseparable.

2. *The need for phosphorus*, or indirectly, the amount of phosphorus ordinarily present in any specific tissue. This will be a gross index of the prospective uptake of P^{32} .

3. *The chemical nature of the phosphorus compound*, that is, whether it is easily ionizable, bound to organic compounds, or in insoluble form, determines in considerable measure the uptake in any specific tissue. An example of an insoluble form is chromic phosphate which is made up in a suspension and after being introduced intravenously is deposited in the reticulo-endothelial system of the liver, spleen and bone marrow.

4. *The permeability of the cell membrane*. The characteristics of the membrane, the pH of the cell environment, and other factors undoubtedly contribute to the transfer of phosphorus into the cell.

If, theoretically, all tissues of the body, normal and neoplastic, had the same type of phosphorus containing constituents and the same turn-over rate, the use of P^{32} would be entirely without rationale and would correspond to external spray irradiation with X-rays. It is well known and well established, however, that normal cells of various tissue structures have different metabolic and turn-over rates and different phosphorus requirements. When P^{32} is introduced into the body, differing levels of radioactive phosphorus will thus occur in the different tissues. In addition to this, tissues in which replacement is rapid (thus producing a rapid metabolic uptake of all the elements and compounds which enter into the constituent of the cell) approach equilibrium much more rapidly than cells of lower activity and thus

achieve a higher level of radioactive phosphorus and a correspondingly higher level of radiation than the less active cell.

If we assume a constant amount of P^{32} in the vascular and intracellular fluid, then the tissue cells in any organism will have the same specific activity of P^{32} (the ratio of active to inactive phosphorus) when the organism finally reaches equilibrium. Before equilibrium is reached, however, those tissues having the more rapid metabolic rate and turn-over will contain more P^{32} or, more precisely, a higher specific activity of P^{32} will exist. If no difference in the metabolic and turn-over rate exists, then the rate at which equilibrium is approached is the same for all tissues and specific activity is the same for all tissues at all times.

In the actual therapeutic application of radioactive phosphorus certain information must be available for orientation in this means of therapy.

1. Information about the pertinent characteristics of the radiation from P^{32} and its radioactivity constants is essential.

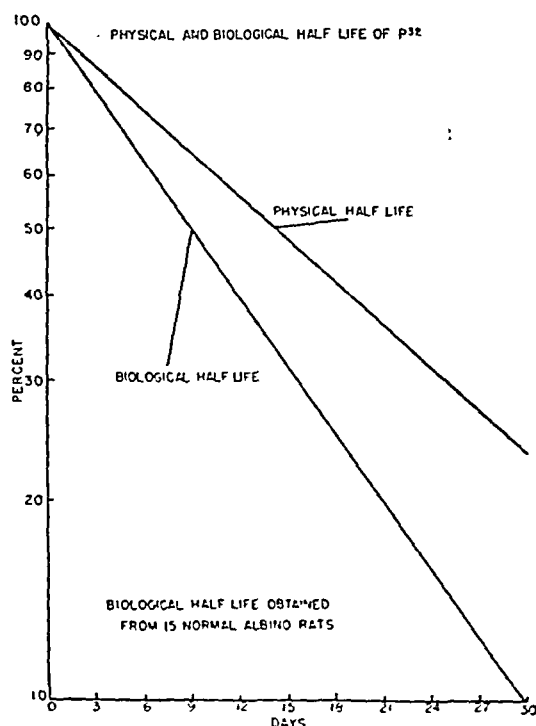


FIG. 1.

The biological half-life is valid only after the third day, at which time 62% of the injected dose was retained. It is a composite curve of the amount excreted plus the decay of the P^{32} .

2. The known phosphorus stores and requirements of the various tissues of the body should be known. The amount and kind of phosphorus-containing compounds is important and the distribution of nuclear proteins, phosphatides, and inorganic phosphate is very helpful.

3. The turn-over rate or metabolic activity of specific normal tissues as well as neoplastic tissue should be evaluated if possible.

4. The rate at which radioactive phosphorus disappears from the body in the excreta should be ascertained. Reference is occasionally made to the biological half-life which is a composite of the rate of loss from the body together with the rate of loss by natural decay (see Figure 1). Obviously this is important in deciding about dosage determinations.

5. The biological effects of the radiation from P^{32} must be appreciated. This can be roughly divided into (a) lethal effects, (b) specific effects on sensitive tissues such as depression of the white blood cells in the hematopoietic system, and (c) other effects, such as radiation necessary to produce erythema of the skin, etc.

Characteristics of P^{32} . P^{32} has radiation and disintegration characteristics which are readily adaptable to biological purposes.² The half-life of P^{32} is 14.3 days. It emits a beta particle whose peak energy is 1.69 MEV and which has an aver-

age energy of 0.7 MEV. Thus, the peak range in tissue is about 8 mm. and the average range is approximately 2 mm. Therefore, in a large mass of tissue, the radiation is essentially localized in the tissue in which the phosphorus is deposited.

The dosage calculations of radiophosphorus, or a calculation of the energy absorbed per unit mass of tissue, are easily made and are often converted into roentgen equivalents, since a great deal of biological information is available on external radiation which has been measured by means of this unit. Since the roentgen is defined in terms of the ionization occurring in a fixed mass of air, it is possible therefore to convert energy absorbed by tissue from internally distributed radioactive elements into roentgens, sometimes referred to as roentgen equivalent physical (rep), or roentgen equivalent.

It is necessary to know the distribution of the radioactive element in tissue, that is, the number of microcuries³ per unit mass of tissue, the energy available on the average from each beta particle emitted by phosphorus, and the number of ergs corresponding to one roentgen. (This is derived from the original definition of the roentgen by introducing the energy required to produce an ion pair.) The calculations for one microcurie per gram for complete decay are as follows:

Calculation of tissue dosage in roentgens due to the complete decay of 1 microcurie of P^{32} in gram of tissue

Half-life = 14.3 days

$$\text{Average life} = \frac{14.3 \times 86,400}{\ln_0 2} = 14.3 \times 86,400 \times 1.443 \text{ seconds}$$

$$\text{Average energy per disintegration} = \text{Peak energy} \times 0.4 = 1.69 \times 0.4 \text{ MEV roentgens}$$

$$= \frac{(\text{microcuries})(\text{dis./sec./microcuries})(\text{aver. life in sec.})(\text{aver. energy in MEV})(\text{ergs/MEV})}{\text{ergs/roentgen}}$$

$$= \frac{(1)(3.71 \times 10^4)(14.3 \times 86,400 \times 1.443)(1.69 \times 0.4)(1.6 \times 10^{-6})}{83}$$

$$= 857 \text{ (roentgens for complete decay of 1 microcurie in 1 gram of tissue)}$$

²Radioactive phosphorus was first produced in the cyclotron by E. O. Lawrence by the bombardment of red phosphorus placed in the external target chamber or the bombardment of an iron phosphide probe placed in the deuteron beam (d,p reaction). Since the development of the piles or nuclear reactors now being operated by the Atomic Energy Commission, radiophosphorus is now made primarily from sulfur by the n,p reaction, producing essentially carrier-free P^{32} . It is also possible to produce phosphorus in the pile by the n, gamma reaction. The n,p reaction with sulfur obviously provides carrier-free material.

³ 1 microcurie is 10^{-6} curies. A curie is defined as 3.7×10^{10} disintegrations per second.

Radioassay of P^{32} . Shipments of P^{32} from Oak Ridge are in the form of aqueous solutions of H_3PO_4 at pH 3-5. These samples, properly diluted, are evaporated on a silver disc so as to approximate closely the size and activity of the deposit of the RaD + E standards distributed by the Bureau of Standards. These samples are then counted along with the RaD + E standard and absorption curves run for both. A value of the count at zero absorber is obtained by extrapolating the above curves to zero, allowing for the thickness of the G-M

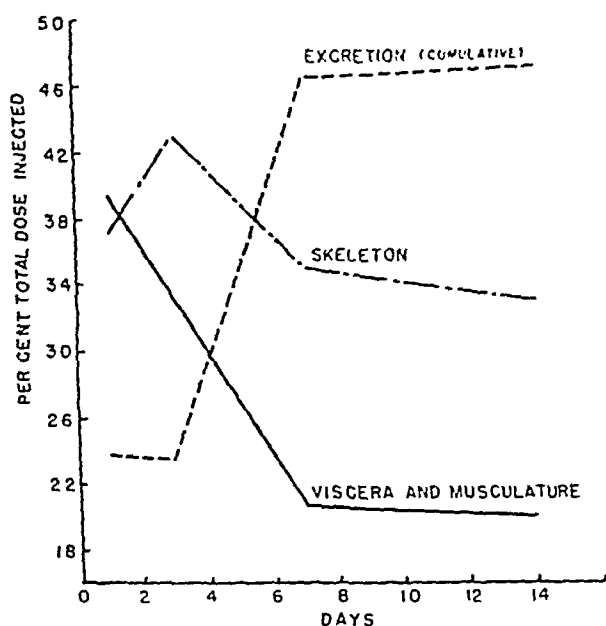
AVERAGE GENERAL DISTRIBUTION OF P^{32} FOR 14 DAYS
IN 16 NORMAL ALBINO RATS

FIG. 2

The curve shows the distribution of retained P^{32} in skeleton and viscera and the amount excreted for intervals throughout 14 days. The amount retained in the skeleton progressively increases beyond the 14 day period.

Phosphorus content of normal tissue. The normal phosphorus stores of the various tissues of the body are an important consideration in the feasibility of treatment with ionizable P^{32} . Since the stores of phosphorus in the tissues generally reflect the need for phosphorus, the amount of P^{32} taken up in tissue will be directly related to this need. Bone, therefore, contains more phosphorus than any other tissue. Figure 2 indicates the character of the distribution of intraperitoneally administered P^{32} in the skeleton and the soft tissues over the first two weeks.

Cohn and Greenberg (15) found that retention of P^{32} per unit fresh weight in various tissues decreased in the following order: bone, liver, in-

testine, heart, kidney, lung, muscle, skin and brain. After 98 days, 92% of the retained phosphorus was present in the skeleton. Studies made on rats in which amounts of P^{32} were considerably greater than tracer doses, gave the distribution as seen in Table I.

The turn-over rate, or metabolic activity of various normal and neoplastic tissue. Some concept of the metabolic activity with regard to phosphorus in the various normal tissues as well as abnormal cellular growths is very important. The greater differential absorption rate in growing tissue as compared with adult tissues has been shown in tissue culture by Bulliard *et al.* (16). Comparison of the uptake of P^{32} of neoplastic and normal tissues has been made by Kenney *et al.* (17) and by Lawrence and his associates (18-21). It has been demonstrated quite conclusively that more P^{32} is present in the rapidly growing tissue than in

TABLE I
Per cent of intraperitoneally injected P^{32} present in
various tissues of albino rats

Organ	1 day	7 days	14 days
1. Skeleton	45.0	41.5	34.2
2. Muscle	19.4	10.8	16.2
3. Digestive Tract	14.4	2.5	4.2
4. Liver	9.2	1.3	1.8
5. Skin	5.4	1.4	2.0
6. Brain	0.13	0.20	0.20

normal tissue, and particularly high in neoplastic tissue. Forssberg and Jacobsson (22) found in their studies of animals with intravenously labeled phosphate that, in the case of transplanted Brown-Pearce tumors, the metastatic tumor took up as much P^{32} as other active organs (spleen, liver, kidney) and about five times as much as less active tissue (diaphragm, omentum, lymph nodes, pleura). Forssberg (23) investigated the uptake of P^{32} by bone metastases and found that these would take up much larger amounts of P^{32} per gram of wet weight than a corresponding weight of normal bone. The work of Marshak (24) in comparing the uptake of P^{32} in the nuclei and cytoplasm of normal and malignant cells indicates that the malignant cells not only accumulate more P^{32} than the normal cells, but that the relative proportion of administered phosphorus in the nucleus as compared to the cytoplasm is much greater in the malignant cell than in the normal cell. This is

tube window and additional air absorber. By comparing the zero absorber counts of the P^{32} and the RaD + E standard, an absolute millicurie value is obtained for the P^{32} . This value must be corrected, however, for the difference in backscattering from silver of the beta particles of P^{32} and the RaD + E standard. This correction, when applied, reduces the above absolute millicurie value by 7%. Results obtained by this method generally check with the assay of the Oak Ridge National Laboratories values to within 5%.

true not only for malignant cells but for rapidly multiplying normal cells.

Studies of the distribution of P^{32} taken up in the nucleo-protein, phosphatide, and acid-soluble fractions in normal tissues, leukemic tissues, and tumor tissues, indicate that the highest uptake was present in the nucleo-protein of leukemic and tumor cells. The differences between the various phosphatide and acid-soluble fractions were not remarkable. It is apparent that the nucleo-proteins of leukemic and tumor cells were synthesized more rapidly than those of normal cells of the mouse, and therefore incorporated more P^{32} . Since no important differences exist between the normal phosphorus content of the nucleo-protein fractions of the various tissues, the specific activity of the nucleo-proteins of the leukemic and tumor cells are therefore higher than that of normal cells.

Rate of loss from the body. Information on the rate at which material is lost from the body is vital. In order to determine the effectiveness of therapy it will be necessary to determine the amount of material actually distributed in the tissues. There are considerable discrepancies between various individuals in the amount retained. It is possible, however, to collect the excreta of patients during the treatment period and ascertain precisely the amount distributed in the body. The information which is gained in this way is difficult to apply in a practical manner. In general, it has been found

that patients excrete 5% to 25% of the administered P^{32} in the first four to six days when given intravenously, and 15% to 50% in the first four to six days when given by mouth (25). It is generally recognized that the retained dose of the material given by mouth is 75% of that given intravenously. Figure 3 shows corollary data which have been obtained in rats, indicating the rate of excretion which may be expected after intraperitoneal administration. At the end of two weeks 46% is excreted, and of the remaining, 20% is in the viscera and musculature, and 33% in the bone. The ratio of bone deposition to soft tissue deposition continues to increase with time. The figure for bone has added significance when one recognizes that bone comprises only 10% of the body weight.

It has been shown that the rate of retention in leukemic patients is higher than in normal for the first few days but it is not improbable that after the first week or ten days the excretion rates in normal and leukemia patients are comparable. In calculating dosage, the biological half-life, which is a combination of the excretion rate and the physical decay rate, is actually more helpful than the physical decay rate alone (see Figure 1).

Biological effectiveness. The biological effectiveness of radiation from P^{32} must be evaluated for each separate system in the body. However, a broad general idea may be obtained from the rate

EQUILIBRIUM AND EXCRETION CURVES FOR P^{32}
FOR 15 NORMAL ALBINO RATS

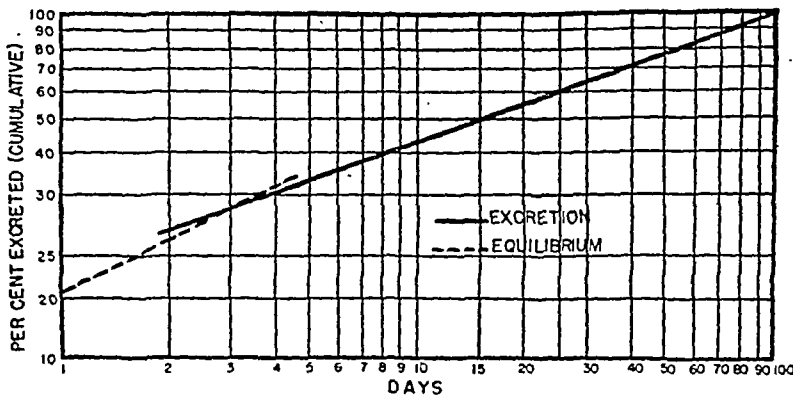


FIG. 3

The rate of excretion changes after the third day. Prior to this, the excretion is definitely more rapid and the curve has been arbitrarily called the equilibrium curve.

15 DAY SURVIVAL CURVE IN
WISTAR STRAIN ALBINO RAT
GIVEN P^{32} INTRAPERITONEALLY

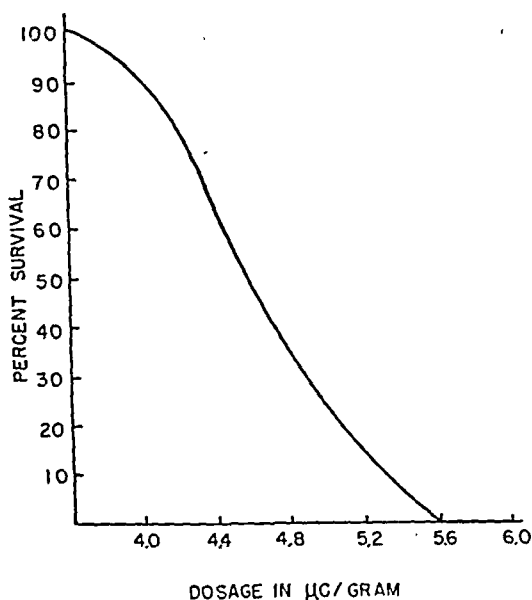


FIG. 4

The LD_{50} for P^{32} given intraperitoneally is approximately 4.5 microcuries per gram in the albino rat (for a 15 day period).

of survival and in Figure 4 a survival curve is shown for rats having received P^{32} intraperitoneally. The LD_{50} for a 15 day period is about 4.5 microcuries of administered P^{32} per gram of body weight. These animals die of severe leukopenia, thrombocytopenia and anemia. There are numerous foci of infection and severe hemorrhagic manifestations throughout the body. Observations have been made with regard to the total maximum depression in the white blood count. Increasing doses show an increase in the total maximum depression. We have found in rats that 0.7 microcuries per gram of body weight produces a maximum depression of about 50% occurring in ten days.

Studies have been made of erythemas produced on the skin by Osgood *et al.* (26). The dose to produce a threshold erythema was found to be 34 microcurie hours per square centimeter.

CLINICAL RESULTS WITH P^{32}

In the past two years, over 50 patients have been treated with radioactive phosphorus at the University Hospitals of Cleveland. In this number

are included the chronic leukemias (myeloid and lymphatic), primary polycythemia, several acute leukemias in children, a selected group of breast carcinomas which have metastasized widely to bone, and a small number of miscellaneous conditions in which it was thought desirable to observe the effect of P^{32} . There were no cases of Hodgkin's disease or lymphosarcoma since the experience with these diseases is, at best, uncertain when treated by P^{32} and because their response to X-ray, at least in the earlier stages of the disease, is generally good. In addition, the treatment of ordinary uncomplicated lymphoid leukemias with P^{32} has been avoided since the bone marrow, although infiltrated with lymphoid elements, is not hyperplastic and the normal elements of the marrow receive unnecessarily heavy radiation. The six cases of lymphatic leukemia included in this study were those which had failed to respond to previous X-ray or were cases in which the disease was so widespread that the immediate outlook was very poor. This accounts for the fact that all the patients with lymphoid leukemia were dead within seven months following the beginning of P^{32} therapy. All the patients reported here had sternal marrow biopsies showing marked displacement of the normal marrow elements by mature and immature lymphocytes.

As has been demonstrated by others, primary polycythemia show excellent response to P^{32} . The response in this group is followed closely by that occurring in chronic myeloid leukemias after treatment with phosphorus.

The cases of acute leukemia, all occurring in children, in general responded very poorly. One individual, however, received definite beneficial effect with a remission which lasted for almost a year—the patient dying at the end of this period. There were widespread destructive lesions in bone which showed considerable improvement following therapy.

It is our intention here to report in detail the primary polycythemia and chronic leukemias since these have received greatest attention in the literature previously and for which the broadest background exists.

Polycythemia rubra vera. Ten cases of primary polycythemia have been treated with radioactive phosphorus. Table II gives a brief summary of the clinical and hematological data for each pa-

TABLE II

Polycythemia rubra vera

Case no.	Age and sex	Wt.	Duration of disease prior to p ₂ Rx	Spleen	Initial hemogram					Total dose of p ₂	Duration of response	Maximum hematological response					Complications of p ₂ treatment	Present status	Other therapy	Remarks
					RBC	Hct*	Hgb†	WBC	Plate-let†			RBC	Hct	Hgb	WBC	Plate-lets				
1 A. M.	55M	160	2 years	0	6.73	54	128	9,800	Normal	9.0 mc. (28 days)	12 months	5.20	50	98	4,500	Normal	None	Dead 18 months after therapy	Venesection prior	Persistent complaints of angina and shortness of breath. Died of cerebral thrombosis
					5.58	52	110	8,700	Normal	6.0 mc. (7 days)	No follow, for 6 months	3.84	41	90	6,350	Normal	None			
					5.40	52	113	11,600	Normal	9.0 mc. (18 days)	1½ months to death									
2 S. W.	68F	115	15 years	4+	7.31	58	100	42,000	Inc.	6.0 mc. (7 days)	6 months	4.42	41	82	9,000	Normal	GI bleeding following 2nd course. Purpura with low platelets after 3rd course	Living and well 2 years after treatment	Venesection prior	Spleen no longer palpable. Asymptomatic platelets normal with clearing of purpura
					6.42	54	103	11,400	Normal	3.0 mc. (1 day)	Failed to return; 1 year	4.20	41	74	2,000	Dec.				
					8.90	59	102	17,000	Inc.	8.0 mc. (14 days)	4 months to date									
3 F. D.	42F	145	16 years	1+	7.94	53	96	6,200	Normal	9.0 mc. (10 days)	15 months to date	4.76	47	76	6,300	Dec.	None	Living and well 15 months after treatment	Venesection prior	Asymptomatic, spleen no longer palpable
					8.28	51	79	51,000	Inc.	9.0 mc. (10 days)	None	7.93	53	97	12,000	Normal	None	Living and well	Venesection prior, during, and after treatment with p ₂	Failed to respond to 2 courses of treatment. Symptoms not relieved
					7.97	53	97	12,000	Normal	12.0 mc. (18 days)	3 months	6.18	55	105	6,200	Normal				
										12.0 mc. (9 days)	4 months to date	5.20	42	95	7,900	Normal	None	Living and well 4 months after treatment	Venesection during p ₂ therapy	Spleen not palpable. Asymptomatic
5 I. V.	66M	155	1 year	1+	9.42	77	130	10,000	Normal											
										12.0 mc. (15 days)	8 months to date	4.84	45	80	6,500	Normal	None	Living and well 8 months after treatment	Venesection during p ₂ therapy	Spleen not palpable. Asymptomatic
6 J. L.	65M	175	1 year	2+	9.00	69	130	15,500	Inc.											
										12.0 mc. (10 days)	4 months	4.65	43	90	8,500	Normal	None	Living and well 6 months after treatment	Venesection during treatment	One myocardial infarction. Angina relieved after 2nd course
					6.21	57	112	11,500	Inc.	8.0 mc. (6 days)	2 months to date	4.90	49	99	8,600	Dec.				
7 D. S.	57M	140	20 years	1+	5.40	54	115	6,100	Normal			4.05	39	79	1,800	Dec.	Marked depression of platelets with spontaneous purpura	Living and well 5 months after treatment	None	Spleen not palpable; platelets normal one month; purpura cleared; much improved
8 J. D.	77F	95	1 year	1+	6.85	52	90	21,000	Inc.	8.0 mc. (5 days)	5 months to date									
9 P. T.	40M	180	1 year	0	6.25	54	118	10,500	Inc.	9.0 mc. (6 days)	8 months	4.65	45	93	6,800	Normal	None	Living and well 8 months after therapy	None	Asymptomatic
10 H. D.	62M	142	4 years	1+	8.80	57	95	20,000	Inc.	6.0 mc. (7 days)	2 months to death	6.44	44	80	12,000	Normal	None	Dead 2½ months after therapy	Venesection prior	Died of luetic aortitis, cardiac decompensation, and cirrhosis
Averages			6.6 years		7.66	58	108	19,000		9.2 mc. (10.7 days)	7 months to date	5.2	45	87	7,900			8 living 2 dead		

* Wintrobe Method.

† Sahli-Haden Method; 15.4 grams = 100%.

‡ Normal platelets 250,000 to 500,000.

POLYCYTHEMIA RUBRA VERA

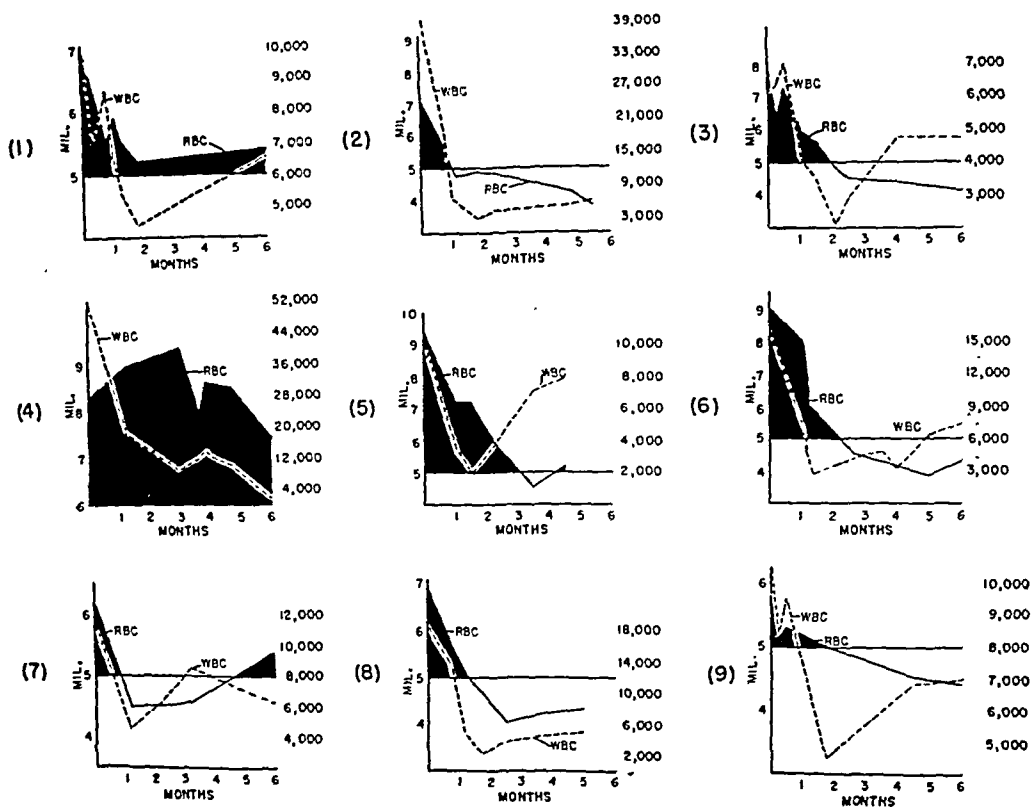


FIG. 5

These curves are the initial hematological responses to P^{32} of the first nine cases. Case 10 was omitted because death occurred before complete hematological response was obtained.

tient, and Figures 5 and 6 give graphically the initial hematological response of the first nine patients and a composite average response of the group.

In Table II it may be observed that all cases had initial red blood cell counts ranging from 6.21 to 9.4 million per cubic millimeter of blood; the hematocrits range from 51% to 77% (Wintrobe method) and the platelets were either normal or increased in number. In the majority of the cases the white cell counts were above normal. In all but two cases a palpable spleen was present. The sternal marrow biopsies showed normal or hyperplastic cellularity.

The response to P^{32} was reasonably prompt. The peripheral blood counts showed definite evidence of response at about one month and the response was maximum in a period of approximately three to four months. Coincidentally, an immediate depression of the white cell count occurred. The average initial white blood cell count was

19,000 per cubic millimeter and leveled off just above 5,000 white blood cells per cubic millimeter after treatment (see Figure 6). In two of our cases, marked depression of the platelet count was demonstrated. This was accompanied by purpura and spontaneous dependent ecchymosis (Cases 2 and 8). In Case 2, this occurred after the third course of phosphorus. After the initial episode, neither patient showed further evidence of hemorrhage and the thrombocyte counts returned to essentially normal levels. Two cases (Nos. 1 and 4) failed to respond adequately to P^{32} . The reason for failure in these two cases is obscure. The diagnosis of primary polycythemia in Case 1 may have been in error. Since chronic lung disease was present, it is possible that this may be a secondary polycythemia.

To date, the duration of remissions in the eight cases responding favorably ranges from four to 15 months with an average remission period of approximately seven months. However, we have

POLYCYTHEMIA RUBRA VERA

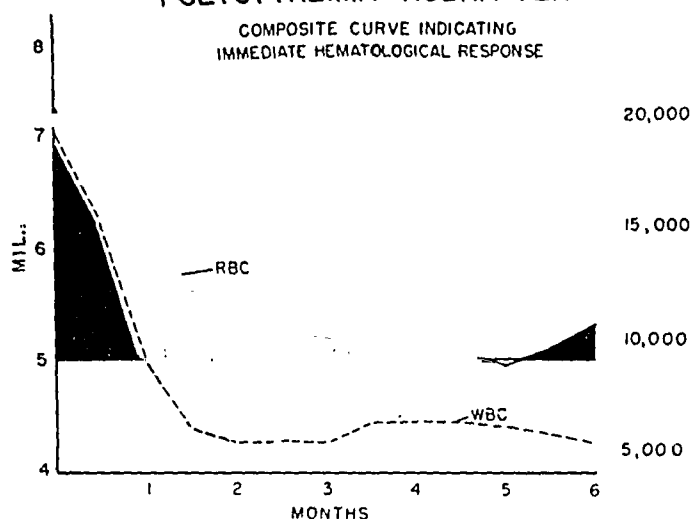
COMPOSITE CURVE INDICATING
IMMEDIATE HEMATOLOGICAL RESPONSE

FIG. 6

Case 10 was omitted from the composite curve. The patient died two and one-half months after therapy was instituted. The average maximum response in the red blood count is obtained in five months, as shown by this curve.

not yet followed these cases long enough to have a definite opinion on the length of the remission period. The indications are that these intervals will be comparable to those observed by others.

The criteria for selecting cases have concerned themselves with evidence of the typical triad of polycythemia, that is, megakaryocytic, erythroid and myeloid hyperplasia. This was present in all the cases. Cases showing less than 6 million red blood cells per cubic millimeter were not treated.

Dosage. The dosage levels for polycythemia have not been rigid or fixed. At present, a dose of 100 to 150 microcuries per kilogram administered over a period of ten days to two weeks appears to produce a satisfactory remission in the majority of cases. Occasionally the administration has been continued over a longer period of time. In general, it has been our tendency to treat polycythemias cautiously since it is not possible to gauge the dosage from the hematological response until approximately three months have elapsed. For this reason it is found necessary to repeat the course in some of the cases after this period of time.

Chronic myeloid leukemia. To date, 15 cases of chronic myeloid leukemia have been treated with P^{32} . Table III gives the clinical and hematological course for each patient. Of the 15 cases,

two failed to respond to treatment with P^{32} in what was considered adequate doses. In Case 10, treatment was stopped because of marked depression of the platelets. At the time of treatment this individual also exhibited a blastic phase of the disease. (It is well known that in this stage the disease becomes refractory to any form of therapy.) The reason for failure in Case 13 is not clear. Since marked splenic enlargement existed, P^{32} therapy was stopped and X-ray given to the spleen with satisfactory results. In the remaining 13 cases, immediate and excellent response was obtained in the white blood cell count and associated with this, a rise in the red cell count was noted in most cases. As is shown in Table III, the maturation of the cells definitely changed, with a clear-cut decrease in the immature cells in practically every instance following treatment. (Arbitrarily we have considered those cells ranging from the early blastic stages to and including the myelocytic stage as immature.) It may also be seen that the average initial white blood cell count was 170,000 per cubic millimeter, ranging from 69,000 to 299,000, and following treatment a composite of the maximum response showed a drop to 17,000 cells per cubic millimeter of blood. The initial red blood cell count was 3.1 million per cubic millimeter and at the end of three months the average red blood cell count rose to 4.1 million cells per cubic millimeter.

In our series of cases, the platelet counts were relatively unaffected during the first course of treatment. This observation agrees with those of Warren (7), who stated that the megakaryocytes showed little damage with moderate dosage. However, subsequent courses tend to depress the platelet count and the rate of decrease in the platelets serves as an important criterion for the continuation of treatment. Figure 7 shows graphically the initial response of the white and red cell count for each patient. It is noted that a uniform reduction of the white cell count occurs promptly as well as a clear-cut increase in the red cell count. Figure 8 is a composite curve of the initial response to P^{32} .

A gradual reduction in the size of the spleen occurred in all 13 cases. In several the decrease was dramatic. Anorexia and weakness, the most common and distressing symptoms, disappeared at

TABLE III
Chronic myeloid leukemia

Case no.	Age and sex	Duration of disease before pa Rx	Physical findings			Initial hemogram			Total dose of pa	Duration of response	Maximum hematological response				Complications of pa therapy	Present status	Other therapy	Remarks
			Spleen	Liver	RBC	WBC	Platelet*	Differential ma- ture†			RBC	WBC	Platelet	Differential ma- ture†				
1 R. J.	34F	4 years	1+	0	2.99	85,000	Increased	89% 11%	12.5 mc. (53 days)	12 months	4.24	7,500	Normal	94% 6%	None	Living 21 months after pa Rx	Arsenic prior, urethane last 8 months	Spleen decreased
2 H. K.	51F	2 months	4+	0	3.53	158,000	Increased	68% 30%	9.3 mc. (20 days)	2 months	4.12	22,300	Normal	75% 25%	None	Living 22 months after pa Rx	None	Spleen decreased
3 G. H.	50F	5 years	4+	0	2.29	209,000	Normal	55% 45%	13.0 mc. (42 days)	2 months	2.96	17,800	Normal	85% 15%	None	Dead 11 months after pa Rx	X-ray, arsenic, prior and after. Urethane after	Generalized military Tbc. Spleen decreased to 1+
4 B. C.	69M	4 years	4+	3+	2.93	77,300	Increased	80% 20%	8.0 mc. (27 days)	6 months	3.98	17,000	Normal	96% 4%	None	Dead 18 months after pa Rx	None prior. Arsenic urethane last 12 months	Spleen decreased to 1+
5 P. P.	49F	3 months	4+	0	2.71	263,000	Increased	30% 70%	11.1 mc. (42 days)	3 months	4.26	15,000	Normal	59% 41%	None	Dead 13 months after pa Rx	X-ray 2 months before death	Pt. admitted in stage of acute blastic leukemia 2 months after last course of therapy
6 H. Ko.	49F	6 months	4+	0	3.88	306,000	Increased	80% 20%	14.0 mc. (57 days)	7 months	4.80	9,500	Normal	95% 5%	None	Living 1 year after pa Rx	None	Spleen not palpable
7 H. M.	29M	3 1/2 years	0	0	1.93	153,000	Increased	54% 46%	8.0 mc. (23 days)	5 months	4.80	6,600	Normal	90% 10%	None	Living 8 months after pa Rx	X-ray prior	Pt. had spontaneous hematomas before Rx
8 M. G.	43F	6 months	3+	0	2.94	177,000	Normal	60% 40%	6.0 mc. (9 days)	2 1/2 months	2.56	63,000	Normal	64% 36%	None	Dead 6 months after pa Rx	None	Spleen decreased to 1+. X-ray to lymph node
9 A. G.	46F	2 years	4+	2+	2.09	148,000	Decreased	55% 45%	10.0 mc. (25 days)	1 month	2.63	26,000	Decreased moderate	55% 45%	Low platelets, no purpura	Dead 3 months after pa Rx	None	Pt. had tonsillar abscess and septicemia
10 A. L.	44F	4-5 years	3+	1+	2.42	285,000	Increased	25% 75%	22.0 mc. (68 days)	None	2.34	212,000	Decreased slight	25% 75%	Low platelets, no purpura	Dead 6 months after pa Rx	X-ray prior, arsenic after	Pt. failed to respond to any treatment
11 H. M.	43M	6 years	2+	0	4.18	122,000	Decreased	81% 19%	9.2 mc. (25 days)	3 months	3.69	11,700	Normal	81% 19%	None	Dead 6 months after pa Rx	X-ray prior, arsenic prior	Spleen decreased to 1+
12 M. L.	43F	5 years	2+	0	4.03	69,000	Normal	84% 16%	13.5 mc. (41 days)	6 months	4.21	17,000	Normal	85% 15%	None	Dead 12 months after pa Rx	X-ray prior and after pa	Spleen decreased to 1+. X-ray to inguinal nodes
13 F. S.	53M	2 years	4+	4+	3.06	161,000	Normal	97% 3%	9.0 mc. (21 days)	None	2.96	165,000	Normal	96% 4%	None	Living and well	X-ray prior and after pa	Pt. responded to X-ray. Maintained on arsenic
14 M. H.	21F	2 years	4+	0	2.77	275,000	Increased	72% 28%	16.5 mc. (65 days)	2 months to date	3.93	18,000	Normal	89% 11%	None	Living and well	X-ray prior	Spleen not palpable
15 K. B.	31F	1 1/2 years	3+	0	4.06	78,700	Increased	90% 10%	8.0 mc. (21 days)	1 month to date	3.76	9,200	Normal	99% 1%	None	Living and well	Arsenic prior	Spleen not palpable
Average					3.10	170,000		69% 31%	10.7 mc. (34.6 days)	4.5 months	3.81	17,000		83% 17%		7 Living 8 Dead		

(Note: Cases 10 and 13 are omitted in average tabulations.)

* Normal platelets equal 250,000 to 500,000.

† Immature includes early blastic stages to and including myelocytic stage. Mature includes metamyelocytes to and including segmented neutrophils.

a comparatively early date. The duration of remissions ranged from two to 12 months with an average of about 4.5 months.

In studying the cases it appears that the treatment of chronic myeloid leukemias with P^{32} is highly satisfactory, but that treatment solely by this means for all relapses is of dubious wisdom and perhaps to be discouraged. In those cases with

splenic enlargement which do not respond, or at best respond poorly to P^{32} , it is often advisable to treat the spleen with X-ray when a relapse occurs. It is our strong impression that the continued and persistent use of P^{32} for every relapse will lead to a hyperplastic bone marrow in a great percentage of cases. It is probably advisable to intersperse other forms of therapy, such as localized X-ray, or ar-

CHRONIC MYELOID LEUKEMIA

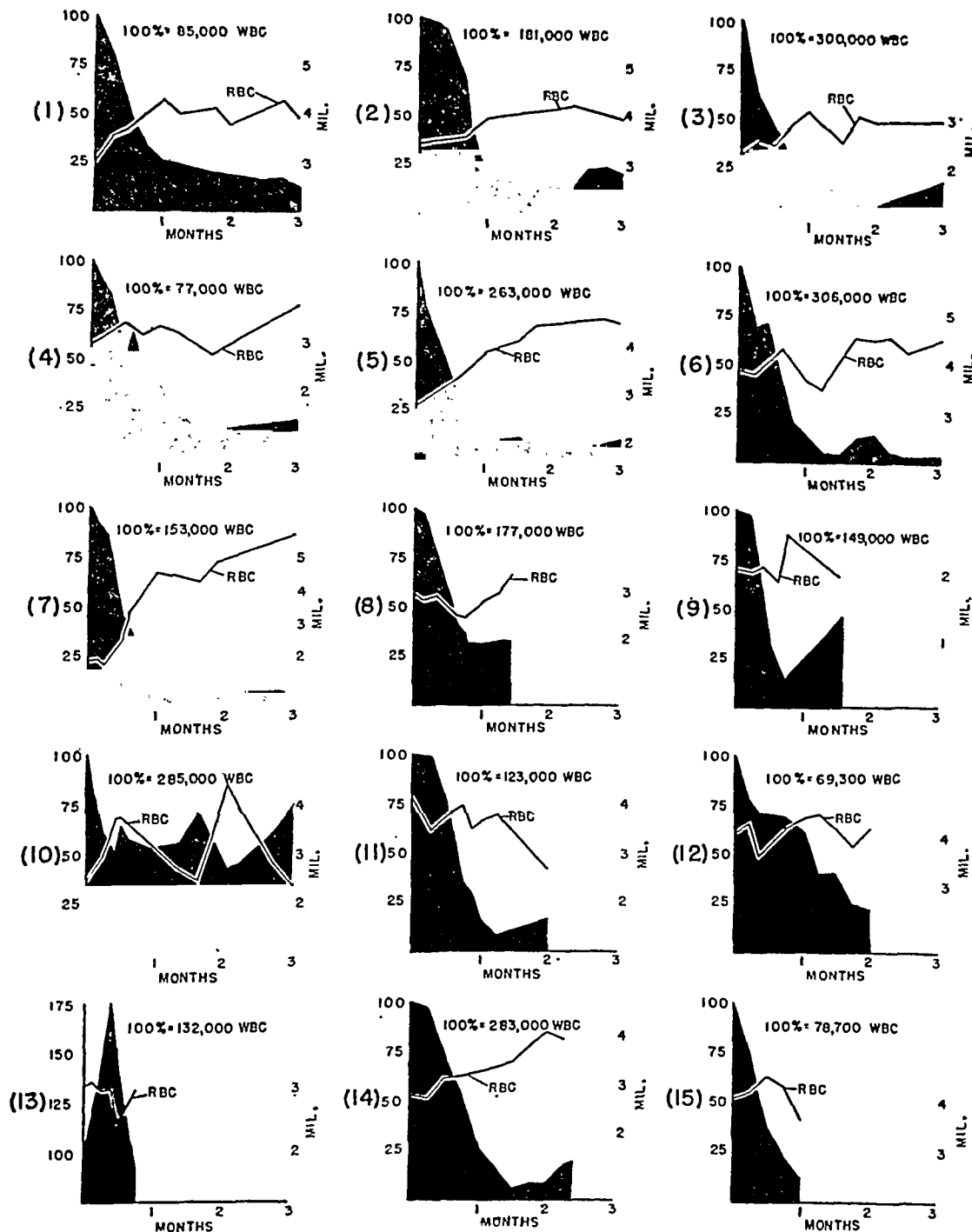


FIG. 7

These curves show the initial hematological response to P^{32} in all 15 cases.

CHRONIC MYELOID LEUKEMIA

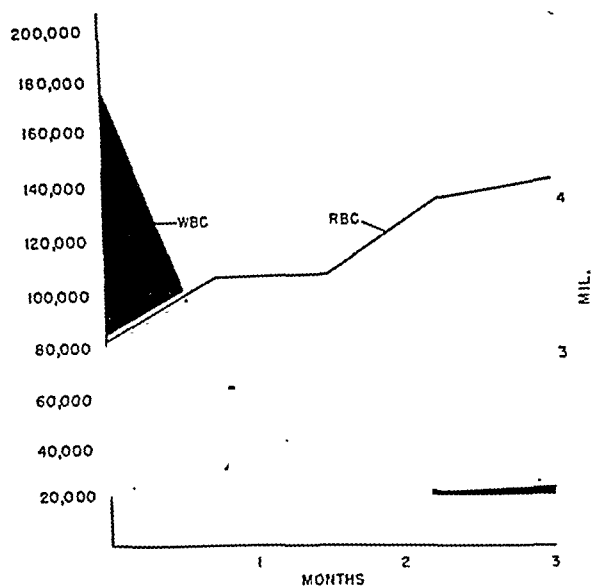
COMPOSITE CURVE INDICATING
IMMEDIATE HEMATOLOGICAL RESPONSE

FIG. 8

Cases 10 and 13 were omitted from this curve. These cases show no response to treatment.

senic (if it proves effective), to increase the intervals between periods of P^{32} therapy.

The criteria for selecting patients have been based upon:

1. *The level of the white cell count.* No cases having counts of less than 20,000 white blood cells per cubic millimeter of blood were accepted for treatment.

2. *The thrombocyte level.* The number of thrombocytes is very important. The patients with low platelet counts were generally treated cautiously or not accepted for treatment. If the platelet count appeared to drop rapidly, treatment was often discontinued for a short interval. During the first course unusual thrombocyte depression does not appear to be a problem, but on re-treatment for subsequent relapses the thrombocyte level must be carefully observed.

3. *Character of the bone marrow.* We have been insistent that the bone marrow show definite hyperplasia before treatment was given.

4. *Maturation of the white cells.* The degree of immaturity to the present has in no way affected our selection of cases for treatment, but if

the number of immature cells are unusually high, one must always consider the approach of an acute blastic phase of the disease which is known to respond poorly.

Of the 15 cases treated with P^{32} , seven are living. The average duration of life in those patients that have died is 3.5 years. This corresponds favorably with the duration of life in a recent report made by Lawrence (14).

Dosage. Again, no hard and fast rules have been applied. It has been found that doses of 1.5 to 2 mc. given at weekly intervals usually produce the desired response after administration of a total dose of 8 to 12 mc. When the white blood cell count approaches 20,000 per cubic millimeter, treatment has been discontinued. As noted by Lawrence (14), it is probably unwise to bring the white blood cell count to normal levels since an effort to depress the white cell count to a normal level requires additional phosphorus and the risk of thrombocytopenia, anemia and serious leukopenia is increased. In the 13 cases which showed satisfactory response, the average dose required to effect hematological remission was 10.7 mc. over a period of 34 days. If one should wish to use a single large dose, there is probably enough experience accumulated in the past ten years to permit treatment of these patients in this manner, but the distribution of P^{32} over a period of a month permits integration of the white blood cell response, and fractionation of the dosage is unquestionably the preferred method of treatment.

Chronic lymphatic leukemia. As has been stated before, the rationale for treating this form of leukemia with P^{32} is not clear-cut. In the majority of cases, although leukemic infiltration is present in bone, the disease is predominantly in lymphoid tissue. The marrow does not have the hyperplasia of all the cellular elements present in myeloid leukemia and in many cases actual damage of the erythroid and megakaryocytic cells is severe. Treatment with P^{32} in most cases serves to depress a bone marrow which appears to have already suffered damage from infiltrating lymphocytic elements. The six cases of chronic lymphatic leukemia selected for treatment, as stated previously, had become resistant to X-ray or were approaching a terminal state. It is doubtful that any form of therapy would have been efficacious.

Table IV gives the clinical data of these six patients. All patients were dead within seven months after P^{32} was started. These cases all had diffuse infiltration of mature and immature lymphocytes on bone marrow biopsies. In all but two cases the initial thrombocyte counts were reduced. (Generally, low initial thrombocyte counts are contraindications to treatment with P^{32} , but in these cases it was felt that an attempt should be made to produce remission no matter how temporary.) Three of the patients were transfused during treatment. The white blood cell count per cubic millimeter of blood ranged initially from 111,00 to 719,000 with an average of 376,000. After treatment the white cell count averaged 78,000 per cubic millimeter, indicating a fair hematological response to P^{32} . As was anticipated, in a number of the cases treatment was stopped because of purpura or extremely low platelet count. It is doubtful whether P^{32} alone contributed to the purpura, since two cases had initial hemorrhagic tendencies prior to therapy.

Case 2 is of interest in one respect which deserves mention. A marked hemolytic phenomenon which was present initially showed dramatic cessation following therapy with a concomitant and unusual response in the white cell count which dropped from 216,000 to 8,000 in only 14 days, after a total of 6 mc. of P^{32} .

Widespread metastases to bone arising from breast carcinoma. Fourteen cases of widespread carcinomatosis of bone from primary breast carcinoma have been treated with what may prove promising results. These cases are to be reported elsewhere and are, therefore, not included in detail in this report. The rationale for treating these cases is based on a well-established fact that malignant tumor cells have a greater P^{32} uptake than most normal cells and that selective uptake of phosphorus in bone is large compared with other tissues, thus producing long-continued selective irradiation of bone. The most striking feature is the relief of bone pain following the treatment. It is not possible at this time to have any definite opinion as to increase in longevity of these patients. In at least one case there was marked deposition of calcium in destructive lesions demonstrated by X-ray. In others the osteoclastic process appeared to be arrested. In all these cases the dosage of P^{32} was pushed to considerably higher levels

at the risk of producing anemia, thrombocytopenia and severe leukopenia. From 18 to 20 mc. were administered in three to four weeks—two cases showing severe purpuric manifestations which subsequently improved. All cases had a moderate to marked depression of the white blood cell count which gradually returned to levels approaching normal after three months.

Treatment of this type with comparatively large doses begins to assume a heroic aspect, but the gravity of the process may justify such an approach if the therapy has promise of any beneficial effects.

DISCUSSION

There is ample evidence experimentally and by empirical clinical application in chronic leukemias and polycythemias that radiophosphorus is an important and effective means of treating these conditions. The cases of polycythemia and leukemia reported here corroborate the promising findings reported by others.

It appears that polycythemias respond unusually well to P^{32} but it is well to remember that this disease is a long-standing chronic process which can be controlled by venesection, although with some difficulty. The apparent superiority of P^{32} over other methods (X-rays, phenylhydrazine and venesection) must be weighed against the possible complications of P^{32} treatment. These are possible thrombocytopenia, leukopenia and anemia. It is still difficult to say what the eventual long time results may be with continued P^{32} application. It appears true that the duration of the remissions, the lowered incidence of the complications of the disease, and the hematological response are quite striking and superior to other means of therapy. It may well be true that P^{32} is the treatment of choice.

In the case of chronic myeloid leukemia the response is prompt, the remissions are of considerable duration and the complications, at least with moderate dosage, appear to be minimal. It is also clear, however, that it is not possible to eradicate the disease with radiophosphorus and an effort to destroy every vestige of leukemic infiltration would irreparably damage the hematopoietic system and perhaps other systems. It is therefore, unwise, as noted previously, to make any such attempt and the use of P^{32} should be limited

TABLE IV

Chronic lymphatic leukemia

Case no.	Age and sex	Duration of disease prior to Rx.	Physical findings			Initial hemogram				Maximum hematological response				Complications to Pa therapy	Present status	Other therapy	Remarks
			Spleen	Liver	Nodes	RBC	WBC	Platelet*	Differential	Total dose of Pa	Duration of response	RBC	WBC	Platelet	ma-ture†	immature†	
1	51M	5 years	3+	0	2+	3.50	239,000	Dec. Moderate	100% 0%	11.5 mc. (34 days)	4 months	4.35	25,000	Dec. Marked	100% 0%	0%	Purpura prior to treatment; Spleen and nodes decreased in size
2	70M	6 months	2+	0	0	2.00	106,000	Dec. Marked	98% 2%	6.0 mc. (18 days)	2 months to death	3.00	34,000	Dec. Marked	100% 0%	0%	Hemolytic anemia ceased after second dose of Pa. Patient transfused during Rx
3	54M	1 1/2 years	1+	0	4+	3.71	111,000	Dec. Slight	100% 0%	8.0 mc. (22 days)	3 months	3.69	14,200	Dec. Moderate	100% 0%	0%	Symptomatic improvement. No change in size of spleen or nodes
4	51M	3 years	2+	2+	2+	3.00	576,000	Dec. Moderate	100% 0%	16.0 mc. (50 days)	3 months	2.79	52,000	Dec. Marked	100% 0%	0%	Transfused during Rx. Symptomatic improvement. Spleen and nodes smaller
5	55F	14 months	2+	2+	2+	2.26	396,000	Dec. Moderate	96% 4%	11.0 mc. (28 days)	Uncertain response 2 months to death	3.50	149,000	Dec. Marked	96% 4%	4%	Uncertain response
6	61F	8 months	3+	3+	3+	3.09	719,000	Normal	96% 4%	18.0 mc. (45 days)	Partial response 3 months to death	2.07	224,000	Dec. Slight	94% 6%	None	Partial response to WBC Spleen and nodes unchanged
Average																	All dead

* Normal platelets 250,000 to 500,000.

† See explanation, Table III.

to inducing remissions with as little phosphorus as possible. The impression is strong that continued application of P^{32} for every relapse with amounts required to bring the leukocyte count to low levels would undoubtedly severely injure the bone marrow and would result in an aplastic marrow in many cases. The use of radioactive phosphorus has several advantages over X-ray in that:

1. Patients have no radiation sickness.
2. The administration of radiophosphorus is simple.
3. It may have the theoretical advantage over X-ray in that phosphorus tends to seek out the most active cells and also selectively irradiates the bone marrow which in the case of chronic myeloid leukemias is, in most instances, the most important site of the disease.

P^{32} cannot be employed without a thorough knowledge of the effects of its radiation, the character of its distribution in tissue, the consequences of possible over-dosage, and clinical course of the patient. Although P^{32} has been used for over ten years, it is not yet completely removed from the realm of experiment. A great many hiatuses exist in the information concerning the effect of P^{32} .

In the case of chronic lymphatic leukemias, satisfactory results have been reported by others, and radiophosphorus is unquestionably useful in this disease. It is our opinion, however, in reviewing the appearance of the bone marrow and the general character of response of patients treated with P^{32} , that lymphatic leukemias should be spared the intensive radiation of the bone marrow which occurs with P^{32} . The appearance of the bone marrow in this disease generally indicates that hyperplasia is not present and that the erythroid and megakaryocytic elements may actually be damaged.

The application of P^{32} for widespread malignancies must be considered purely experimental. In general, treatment of these conditions with P^{32} has been poor.

The dosage of P^{32} is still not fully standardized. The amount of phosphorus administered is gauged primarily by the clinical response, by the size of the patient, by the extent of the disease and by careful observation of the known complications—leukopenia, anemia, thrombocytopenia. In general

we have, as noted above, used a routine of approximately 2 mc. a week to a total of 10 to 12 mc. for the chronic myeloid leukemias and, on the average, a total of 10 mc. in a 12 day period in the case of polycythemia. This works out to be about 150 microcuries per kilogram in a ten to 14 day period and a comparable amount in a 30 day period for chronic myeloid leukemias. In the case of chronic leukemias, fractionation is the preferred method of treatment since it permits integration of the hematological response into the treatment. In the case of polycythemia, fractionation over the period of time required for maximum responses (three or four months) would be impractical and we have, therefore, chosen to administer the dose, as have others, over a short period.

SUMMARY

1. The rationale for using P^{32} as an internally distributed source of radiation and its selective uptake by various normal and tumor tissues has been reviewed. The basis for this, which rests on experimental, biological and clinical work, is discussed.

2. The pertinent information necessary to apply P^{32} effectively is noted. Experimental and clinical data in this regard are presented. The physical characteristics of P^{32} and its behaviour biologically are included.

3. Primary polycythemia and chronic myeloid leukemias are effectively treated with radioactive phosphorus. Ten cases of primary polycythemia and 15 of chronic myeloid leukemia are reported, confirming the observations of others. P^{32} has also been used for a variety of malignant conditions other than leukemia. None of the responses have been striking. Mention is made of possible beneficial application to widespread metastases to bone from carcinoma of the breast.

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RADIOACTIVE PHOSPHORUS IN STUDIES ON THE METABOLISM OF NORMAL AND NEOPLASTIC TISSUES

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Since, in the past year, there have appeared one book and three reviews covering wholly or in part the subject matter indicated above (1-3), this review will confine itself to those aspects which have not been fully presented or in which the interpretations differ from those of the present reviewer. The retention of P^{32} by various normal and neoplastic tissues has been well summarized as has phospholipid metabolism and will not therefore be considered here (4, 5).

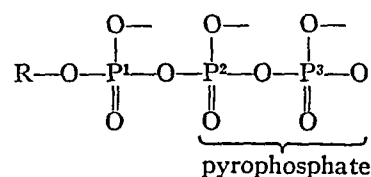
Phosphate esters and carbohydrate metabolism

The rate of penetration of phosphate ion into muscle was shown to be relatively slow, so that the specific activity (P^{32}/P^{31}) of the inorganic phosphate (IP) within the cell was for a long time much lower than that of the extracellular IP and was shown to be a function of temperature (6). Assuming that the extracellular space for phosphorus and sodium were the same, Hevesy and Rebbe estimated that IP is incorporated into organic phosphate at the rate of $21 \mu\text{g/gm tissue/day}$ (7). Direct determination of extra- and intracellular radioactive inorganic phosphate (P^*O_4) has been made by equilibration of heart muscle slices in a medium containing (P^*O_4). The rate of penetration was $1.7 \mu\text{gP/gm tissue/min.}$ at 37°C and about $0.3 \mu\text{gP/gm tissue/min.}$ at 2°C (8) which was in agreement with data obtained earlier by Manery *et al.* (9). Equivalence between extracellular IP and that of the medium was reached in 10 to 30 minutes at either 2° or 37° , but the specific activity of the intracellular IP increased with temperature as well as with time (8). The difference in P^{32} entering the cell at 2° and at 37° indicated that cell penetration by PO_4 is not a simple matter of diffusion.

The specific activity of creatine phosphate (CP) was equal to that of intracellular inorganic phosphate while that of the labile phosphate of adenylyl pyrophosphate (APP) was lower. The terminal phosphate of adenosine-triphosphate (ATP) had the same specific activity as IP and CP, while the

remaining labile phosphorus of APP was lower by about one-half. These results were taken as evidence that intracellular IP and not total tissue IP was the source of supply of CP and APP (8). In frog gastrocnemius *in vivo* the specific activity of CP and of the labile P of the pyrophosphate fraction were found to be equal (7). Sacks and Altshuler (10) found a difference in the specific activity of IP and CP of both skeletal and heart muscle and concluded that extracellular and intracellular IP exchange through CP and APP as carriers (10). The interpretation has been criticized since their data were rather variable, since they assumed a fixed extracellular space and since they also assumed equilibrium in both concentration and radioactivity of plasma and extracellular IP (8). Bollman and Flock (11) found that in both resting and exercised limbs of the rat, the P^{32} uptake was the same for both CP and APP and there was no increase in specific activity in the recovery period. These results would be expected if the fractions were in equilibrium with only intracellular IP during the experimental period. Furchgott and Shorr (8) calculated that there is almost complete turnover of labile organic phosphorus in the cell once every two minutes.

The phosphorus groups of ATP may be designated as follows:



In contrast to the rapid turnover in P^2 and P^3 , the turnover rate for P^1 was found to be slow (12-15). Kalckar *et al.* (15) made analyses after perfusing and they found that in skeletal muscle labile organic phosphate turned over at the rate of $25 \mu\text{gP/gm tissue/min.}$ In ATP of rabbit muscle at 37° the specific activities of P^2 and P^3 were the same, while in the frog muscle at low temperature the P^2 was higher. In rabbit liver, pyrophosphate turnover was at the rate of $17 \mu\text{g/gm tissue/min.}$

Hexose monophosphate activity was either higher, lower or the same as the pyrophosphate of ATP, the variability being attributed to the extent of direct formation of phosphorylated sugar from glycogen and IP (15).

Kaplan and Greenberg (16) determined P^{32} uptake in a number of organic phosphorus compounds of rat liver. Instead of specific activity, they used the ratio of P^{32} in the fraction in question to the total acid soluble phosphorus as a measure of the P^{32} distribution in the various components of this fraction. The extracellular inorganic phosphorus was not separated or estimated so that calculations of absolute rates of turnover would be subject to considerable error (8, 15). Their data showed the uptake to be greatest in glucose-6-phosphate and lowest in glycerol-phosphate. The injection of glucose or insulin increased the level of P^{32} in ATP pyrophosphate but not in the hexose-phosphate. The greatest increase was observed after administration of both insulin and glucose. They suggested that the ATP content of the liver was of primary importance in determining the shape of the glucose tolerance curve. (High fat and protein diets decreased P^{32} esterification [16].) Sacks reported an increase in the turnover of hexosephosphate as well as creatine phosphate and adenylypyrophosphate following the administration of insulin (17). In addition, P^{32} uptake by brain tissue *in vitro* is markedly increased on the addition of glucose (18).

In general the results seem to fit the concept of a rapid turnover of phosphorus in the intracellular phosphate ester pool with a much slower turnover of inorganic phosphorus from the extracellular phase to replace phosphorus "leaking" out of the cell.

Nucleic acids and nucleoproteins

The "residual protein" of tissues is that portion which remains after the extraction of the acid soluble and lipid fractions. This has sometimes been called the nucleoprotein fraction although strictly speaking nucleoprotein is only one portion although it may be by far the greatest in this moiety.

The apparent rate of renewal of desoxyribose nucleic acid in normal rabbit liver was found to be very slow (19, 20). However, the turnover in the residual nucleoprotein of normal

liver nuclei was found to be quite rapid (21). Turnover rates for desoxyribonucleic acid (DNA) of several tissues have been calculated assuming equilibrium with intracellular IP where the latter was calculated from the Na^{24} space and the tissue IP so that calculations of absolute rates are subject to the errors previously mentioned (8, 15). The relative specific activities in order of decreasing activity were intestinal mucosa, sarcoma, spleen, liver, kidney. A regressing sarcoma gave the same DNA specific activity as one which was growing (22, 23). It was suggested that dividing or secreting cells may be more permeable to phosphate than others (1). Bone marrow DNA showed a very high specific activity and that for the thymus decreased with increasing age of the animal (24). The rate of P^{32} incorporation in DNA reported by Brues *et al.* (25) was much higher than that found by Hevesy and Ottesen (20) but was probably contaminated with RNA. Brachet (26) pointed out a parallelism between DNA turnover rate and the intensity of the nuclear alkaline phosphatase reaction.

The rate of P^{32} uptake by ribose nucleic acid (RNA) and its apparent turnover rate has been reported to be much higher than that for DNA (25, 27). In liver tissue the ratio of apparent turnover rates for RNA and DNA was found to be 30, in spleen 3 and in mucosa 2. This has been taken to indicate a high rate of actual turnover in the latter two tissues (1, 27). It may be significant that the relative RNA turnover in intestinal mucosa, spleen and liver parallels that for protein synthesis as determined with N^{15} labelled leucine (28).

Although many of the observations on P^{32} accumulation in DNA have been interpreted as indications of actual turnover, evidence had been advanced earlier that such an interpretation may not be justifiable. It has been shown that in tissue which is not mitotically active (normal liver), there is rapid movement of P^{32} containing constituents between the cytoplasm and the nucleoprotein of the nucleus so that the partition ratio remains practically constant from one hour to five days after P^{32} administration. In actively mitotic tissue (lymphoma, sarcoma, regenerating liver), however, there is a continuous rise in the partition ratio indicating movement into nuclear nucleoprotein (largely DNA) but little or none

coming out. Since the tissues to which a rapid DNA turnover has been attributed are also those in which there is rapid cell division, it is apparent that accumulation in DNA and not turnover may be the more appropriate interpretation. It would seem advisable to reexamine data on the apparent dynamic equilibrium obtained with isotopes other than P^{32} to determine the extent to which the data may be explained by cell replacement as compared with molecular turnover.

Nucleic acid turnover in relation to mitosis

Using a simple new method for isolating nuclei, Marshak (21) compared the rate of P^{32} accumulation in mouse liver and mouse lymphoma and in normal and regenerating rat liver with the rates for the nuclei of the same tissues. Initial uptake by normal liver and by normal liver nuclei was greater than that by lymphomatous tissue, but eventually the retention was much greater in the tumor tissue. Although the ratio of P^{32} concentration in liver nuclei to that in the whole liver tissue remained constant over a period of from one hour to five days after P^{32} injection, in the lymphomas studied, this ratio showed a continual increase. Sarcoma 180 gave results similar to lymphoma, while regenerating liver, in contrast to normal liver, also showed increased accumulation in the nuclei. In the nuclei of normal liver 60–70 per cent of the P^{32} was in the residual nucleoprotein with the remainder in the acid soluble and lipid fractions. Approximately the same distribution was found at various times between one hour and seven days after P^{32} administration. In the lymphomas the residual nucleoprotein contained about 95 per cent of the nuclear P^{32} . The data were taken as evidence that in the resting nucleus there is continual replacement of nuclear nucleic acid phosphorus at a rate greater than that required for complete replacement in one day. This is in striking contrast to the observations of Hahn and Hevesy on DNA extracted from tissue. Although there was obvious replacement in "resting" nuclei, with P^{32} in some form being rapidly built into the nucleic acid, and P^{32} containing components of nucleic acid also being rapidly removed to the cytoplasm, the situation in mitotic nuclei differed in that the P^{32} was built into the nuclear nucleic acid with little or none being released to the cytoplasm. Calculations from the rate of P^{32} accumulation in lymphoma nuclei showed that in 27

hours the nucleus had accumulated an amount equal to that initially present, *i.e.*, an amount sufficient to form another nucleus. This was in approximate agreement with data on rate of growth of the tumor from which it was estimated that its weight was doubled in 24 hours (21).

Brues *et al.* (25) found that between three and eight days after P^{32} administration, the specific activities of nuclear residual protein and nuclear nucleic acid were each slightly higher than that of DNA extracted from the whole tissue. RNA had a much higher specific activity than DNA in normal liver but these were approximately the same in regenerating liver. The phospholipid of the nucleus was at all times in equilibrium with that of the cytoplasm, indicating that the low DNA uptake could not be due to exclusion of P^{32} from the nucleus as had been suggested by Hahn and Hevesy. The P^{32} uptake by DNA of a hepatoma was five times greater than in normal liver, but only half as much as in regenerating liver. A correlation between increase in P^{32} incorporation in DNA and mitosis was also reported by Kohman and Rusch (29) who found a greater P^{32} uptake in nucleic acid of hepatoma as compared with normal liver. In the avian nucleated erythrocyte which does not undergo mitosis, the DNA specific activity does not decline until the erythrocytes are destroyed (30).

When P^{32} -labelled chromatin, fat-free chromatin, or that portion of chromatin soluble in 1 M NaCl were given intravenously to rats, the P^{32} was rapidly incorporated into the liver nuclei. Since the chromatin administered was particulate and could, therefore, be expected to accumulate in the liver, comparison was made of nuclear P^{32} uptake as per cent of the total liver P^{32} thus permitting comparison of nuclear P^{32} accumulation independent of the total tissue concentration. Comparison on this basis showed that nuclei accumulated over four times as much P^{32} when the source was fat-free chromatin as when it was inorganic phosphate, and twice as much as when it was phospholipid. This observation was correlated with the marked stimulation of mitosis produced when chromatin was injected into partially hepatectomized cats (31). P^{32} -labelled DNA from Jensen sarcoma was injected as a solution into rats and two hours later a considerable amount of P^{32} was found in the liver. The P^{32} concentration in the liver and kidney were about the same, while in the

spleen it was much lower. Of the liver P^{32} about three-fourths was in the acid soluble fraction (32).

Ahlström *et al.* (32) and Hevesy (1) interpreted the findings of Marshak and Walker as another example of accumulation in the liver of foreign substances injected into the blood stream. However, this cannot be accepted as a correct interpretation since the basis of comparison used, as explained above, eliminates this source of error.

The rapid P^{32} incorporation into the residual nucleoprotein of resting nuclei found by Marshak when compared with the inactivity of DNA observed by other investigators and also the efficiency of chromatin as a P^{32} donor suggested the presence of a component of nuclear residual protein which was unusually active in P^{32} turnover.

Nuclei were isolated from animals that had received P^{32} three hours earlier and it was found that on incubation at $0^\circ - 2^\circ$ very little or no P^{32} , nucleotide or nucleic acid was removed but that at 37° large amounts of polynucleotide of high specific activity were released. In saline the addition of ribonuclease (RNase) doubled the P^{32} released while desoxyribonuclease (DNase) gave only a 50 per cent increase. After digestion with ribonuclease, the addition of the DNase released no more P^{32} than did native nuclear enzymes under the same conditions although very large amounts of unlabelled polynucleotides were liberated by the DNase. The same results were obtained with normal and with regenerating liver. These observations therefore indicated that during the three hours the P^{32} was in the animals, considerable amounts had been incorporated into nucleic acid but none into DNA. When the incubation was carried out in carbonate buffer at pH 7.0 and pH 7.5, the P^{32} released was increased but the amount released in the presence of DNase was less than when no enzyme was added and it was therefore suggested that this enzyme might bind the P^{32} containing substrate although it did not split it. In the buffer all of the P^{32} containing nucleic acid was removed by native enzymes and/or added ribonuclease, the P^{32} not so removed could be accounted for as phospholipid and acid soluble organic phosphorus. The specific activity of the P^{32} containing nuclear substrate was 13 times greater than that of the cytoplasmic RNA. It was concluded that the two are functionally different substances although the substrate material might be the same as that fraction which could be iso-

lated from nuclei by methods used for obtaining RNA, since they both had about the same specific activity. Since the nuclear autodigestion products were polynucleotides, it could be concluded that the nuclear enzymes involved were not the same as ribonuclease. Solubility studies showed that the P^{32} containing substrate differed from desoxyribonucleoprotein. Since P^{32} first appears in this nucleoprotein in mitotic cells and does not appear in DNA until some time later, this material was considered to be the precursor of DNA in such cells. Earlier experiments had shown that in resting cells (normal liver) there was rapid exchange between nuclear and cytoplasmic P^{32} and not unidirectional accumulation by nucleus and DNA as in the case of mitotic nuclei (21). Under these conditions therefore the higher specific activity of the nuclear substrate indicated that in the resting cell it was the precursor to cytoplasmic RNA (44).

X-ray effect on nucleic acid turnover

Marshak (21) showed that low doses (200 r) of X-rays produced an increase in the relative amount of P^{32} accumulated by nuclei of a mouse lymphoma although the total tissue uptake remained unchanged. Since the total tissue P^{32} was not altered, the observations could not be attributed to changes in permeability. The increase in nuclear P^{32} concentration varied with the dose. Since this effect was not observed in normal liver, the phenomenon was considered to be somehow associated with mitosis.

Euler and Hevesy (33) subsequently studied the effect of X-rays on the Jensen rat sarcoma. They found no significant effect with doses of 77–450 r on P^{32} concentration in total nucleic acid of the tumor. At doses of 450–7,000 r, the nucleic acid P^{32} as per cent of the liver IP and plasma IP was much reduced. The results could not be explained as effects on cell permeability since there was a parallel reduction in ratios of nucleic acid P^{32} to IP and to plasma P. This conclusion is valid only if the intracellular IP and the total tissue IP are not very different. A number of subsequent experiments gave further information on the reduction of P^{32} incorporation in DNA of sarcoma and liver, kidney, spleen and intestinal mucosa (22, 34, 35).

As in the case of the mouse lymphoma and liver (21) a correlation between the known inhibition

of mitosis and the X-ray effect on nucleic acid was established. However, there were these important differences. The significant effects observed by the Scandinavian investigators were obtained only at doses between 450 to 2,000 r and were all in the direction of decreasing P^{32} incorporation into DNA. On the other hand, the results with the lymphoma were obtained at doses between 200 and 260 r and were in the direction of increased P^{32} incorporation in the nuclei. With the rat sarcoma there was also marked reduction in the total P^{32} of liver, sarcoma, and plasma as well as of tumor IP following irradiation while in the mouse experiments there was no change in total P^{32} of the lymphoma. Experiments were performed on rats each bearing two tumors one of which was irradiated and the other carefully shielded and it was found that the non-irradiated tumor showed a decrease in DNA — P^{32} uptake almost as great as that of the irradiated tumor (22). The effect on DNA must, therefore, be an indirect one which is somehow produced by an initial systemic reaction. This explanation does not apply however to the results on lymphoma. Marshak has proposed a theory for the mode of action of X-ray which postulates an inhibition of conversion of the nuclear precursor to ribonucleic acid and correlates this inhibition with arrest of mitosis known to be produced (44).

Bone

P^{32} given as phosphate appears rapidly in bone (36, 37). Pecher showed that the relative concentration in bone as compared with soft tissue was lower than that observed with Ca^{45} and Sr^{89} which was anticipated because of the comparatively low Ca content of soft tissues (38). The concentration of P^{32} was greater in the spongy portion of bone and for this reason the specific activity of the epiphysis was at first higher than the diaphysis although, with time, this difference disappeared (39). The initial P^{32} deposition in bone was by ionic exchange with blood phosphate and the solid phase of bone, but this was altered later by the constant resorption and deposition which took place (40, 41). It has been proposed that mineral deposition occurs when a local excess of PO_4 is produced by action of alkaline phosphatase on phosphate esters to the point where the solubility product of bone salt is exceeded (42). A quanti-

tative method has been described for studying the healing of bone lesions by measuring P^{32} and Sr^{89} uptake *in situ* without disturbing the lesion and using the contralateral limb as a control. The ratio of strontium to phosphorus increased as the healing process progressed and it was suggested that this difference might be utilized in differentiation between the proliferating and calcifying stages (43). A decrease in the uptake of P^{32} , Ca^{45} and Sr^{89} by bone in rickets and an increase in their uptake on administration of vitamin D has been demonstrated by several authors.

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THE USE OF RADIOACTIVE IODINE IN STUDYING THE PATHOLOGIC PHYSIOLOGY OF THYROID DISEASE

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It was in May, 1938 that Hertz *et al.* (1) published the first report on biological studies with a radioactive isotope of iodine. By today's standards these first studies were done under the least favorable conditions with an isotope whose half life of 25 minutes limited the studies that could be done. Notwithstanding the limitations put on these investigators they not only reaffirmed certain principles of thyroid physiology, but with other investigators, Hamilton (2), Hamilton and Soley (3), and LeBlond and Süe (4), who began working with radioactive isotopes of iodine in other laboratories at about the same time, they pioneered in a new field of investigating the thyroid. Today we have a small army of investigators bringing together a variety of disciplines, *i.e.*, physics, chemistry, embryology, physiology, histochemistry, pathology and clinical medicine in studying with radioactive iodine and other tools normal and morbid physiology of the thyroid. Many of these investigators are students whose primary interests are related to studies of normal and abnormal function of the thyroid. These students are delighted to have made available so many new technics with which to increase our knowledge of thyroid physiology. Many of the investigators in this field are interested primarily in isotopic methods and their application to studies in biology. They probably have been attracted to this field because of the unique avidity of the thyroid for iodine which makes this an ideal tissue for applying isotopic methods, and for studying the biologic effects of irradiation with such isotopes.

At the present time, it seems quite proper to ask questions such as those posed by Dr. William T. Salter preceding the 1948 Symposium on Radio Iodine at the Brookhaven National Laboratory. "1. How has radio iodine improved our knowledge of iodine metabolism and of the physiology and the therapy of the thyroid? 2. How do the results harmonize with past experience?" The purpose of this paper is to review the contributions made

to our knowledge of human thyroid disease by studies with radioactive iodine.

The earliest studies done at the clinical level by Hamilton and Soley (3) reaffirmed our previous concepts that the human thyroid has an avidity for iodine. These investigators compared the collection by the thyroid of iodine labelled with radio iodine and its excretion in the urine and feces in normal subjects and in patients having various thyroid disorders. They found that an orally administered dose of labelled iodine was absorbed rapidly and could be detected in the thyroid within 20 minutes. In normal subjects 74 to 89 per cent of the dose was excreted during a five-day period with the major portion appearing during the first 24 hours. Two myxedematous patients excreted 91 and 94 per cent in the urine during five days but at a much slower rate than did the normals. Thyrotoxic patients previously treated with iodine excreted about the same amount of iodine as did normal individuals. Fecal excretion of these labelled doses of iodine averaged only about 1 per cent of the dose. In another series of studies Hamilton and Soley (5) recorded the characteristic collection curves obtained by *in vivo* measurements for various thyroid states. The curve that they observed to be typical for normal thyroids was a smooth curve which leveled off to a flat plateau in two days. The initial collection by thyroids of hyperthyroid patients was greater and much more rapid than in normal thyroids but decreased almost as rapidly to a plateau lower than that of the normal thyroids. This phenomenon was also observed in two goiterous hypothyroid children (Hamilton, *et al.* [6]). This rapid decrease to a lower than normal plateau following an increased pickup observed in the goiterous children and hyperthyroid individuals might be attributed to the fact that these investigators were using 14 mgm. of inert iodide as carrier. This concept is supported by their failure to observe the rapid loss of radio iodine from the thyroid when a carrier dose of 0.1 μ g. of sodium iodide was given.

This explanation is further supported by the observations of Hertz *et al.* (7) who administered radioactive iodine with varying amounts of carrier iodide to patients suffering with Graves' disease at varying stages of preoperative treatment with potassium iodide. They observed that the untreated hyperplastic thyroids of Graves' disease collected 80 per cent or more of the labelled iodine if the dose was small. They also observed that with increasing time intervals after the administration of isotopic iodine there was an increasing amount of radioactive iodine in the thyroxine-like fraction of the gland.

Since these comparatively earlier observations, a number of investigators have used isotopic techniques in studying the role of iodine in normal and abnormal thyroid physiology.

Chapman *et al.* (8) have studied the collection of radio iodine by the human fetus. They administered tracer doses of I^{131} 12 to 48 hours before operation to pregnant women who because of organic disease were coming to therapeutic abortion. The intact fetus was fixed in formalin and then sectioned longitudinally, one half was taken for measurements of radioactivity and the other half was examined histologically. They observed that fetuses less than 12 weeks old did not concentrate any measurable amount of labelled iodine. In those fetuses which varied in age from 14 to 32 weeks the thyroids showed an avidity for iodine which seemed to increase with the age of the fetus. It is of interest that these investigators were able to confirm the opinion that the onset of function as measured in this manner is correlated with the appearance of definite follicles containing colloid.

Astwood and Stanley (9) studied the rate of accumulation of radioactive iodine by the thyroid in 70 normal humans. By plotting the counting rate per second obtained at a standard distance from the thyroid against the square root of the elapsed time in minutes they were able to plot a straight line which continued for about eight hours from which they were able to determine an accumulation gradient. In normal humans they found this accumulation gradient to vary between the limits of 1.5 and 36.4 with a mean of 9.3. Myxedematous patients were found to have accumulation gradients of 0.5 to 1.9 and thyrotoxic patients receiving no treatment at the time of testing were found to have gradients which varied between 17.0 and 43.6.

Keating *et al.* (10) followed the rate of radioactive iodine excretion in patients who had received 100 microcuries of I^{131} with 100 μ g. of carrier iodine. By mathematical analysis their urinary excretion curves yielded four quantities: 1. The renal fraction (that fraction of the dose excreted in the urine); 2. The disappearance rate (the proportional rate of disappearance from the blood); 3. The renal excretion rate; and 4. The collection rate (the proportional rate of disappearance into other sites than the kidneys which they interpreted as an index of thyroid collection rate). They found the renal fraction to be greater than normal in hypothyroid patients and less than normal in hyperthyroid patients. The disappearance rates they found to be less in hypothyroid patients and markedly greater than normal in patients having hyperthyroidism. The most significant variation that they observed was in the collection rates which were much less than normal in hypothyroid states and averaged six times the normal in hyperthyroid patients.

These marked variations from normal in the collection of iodine by the thyroid or the urinary excretion of radio iodine in hypothyroidism and in hyperthyroidism have prompted many investigators to use such methods of study as a means of diagnosing abnormal states of thyroid function. Quimby and McCune (11), who used *in vivo* measuring technics, found that children who clinically were euthyroid concentrated and retained in their thyroids about 12 per cent of an administered dose of radio iodine. They found that hyperthyroid children concentrated and retained several fold the value obtained in euthyroid children whereas hypothyroid children concentrated as little as 1 per cent or less of the dose. McArthur *et al.* (12) followed the urinary excretion of radio iodine in a series of patients and observed the urinary excretion of 22 thyrotoxic patients to average 25 per cent; of 30 non-thyrotoxic patients to average 60 per cent. They reported that this method of study was of real value in excluding the diagnosis of Graves' disease in patients having hypermetabolism due to alcoholism, anxiety, compensated hypertensive cardiovascular disease, Parkinsonism, pheochromocytoma and thyrotoxicosis factitia.

Feitelberg *et al.* (13) have described a method for measuring and recording localized collections

of radio iodine. It is of interest that with this method they were able to demonstrate a functioning lingual thyroid.

Werner *et al.* (14, 15), have used *in vivo* methods for determining the per cent concentration and retention of radio iodine by the thyroid. They reported that the uptake by normal thyroid tissue is 20 to 30 per cent of the administered tracer and that any uptake of 40 per cent or more is regarded by them as diagnostic of hyperthyroidism.

Skanse and Riggs (16) reported that with radioactive iodine tracer studies they were able to make the diagnosis of thyrotoxicosis factitia in two patients who had elevated basal metabolic rates and abnormally high serum precipitable iodines. Both of these patients excreted more than 97 per cent of the radio iodine and by *in vivo* measurements were found to concentrate none in their thyroids. Rawson and Skanse (17) have called attention to the fact that thyrotoxic patients who have previously received "priodax" for x-ray visualization of the gall bladder excrete most of the radio iodine given within an indefinite period of time following cholecystography. Such patients, if clinically thyrotoxic, on the basis of elevated serum precipitable iodines and maximum excretion of radio iodine might be confused with patients having thyrotoxicosis factitia. This problem should be a rare one if careful physical examinations are done. In thyrotoxicosis factitia the thyroid should not be enlarged or it may be so atrophic, as the result of the ingested thyroid, as to be impossible to feel, whereas in classic Graves' disease the thyroid can be felt without any trouble by an experienced examiner.

The above studies have provided base lines which have been useful in evaluating the effect on the thyroid of various thyroid-stimulating or thyroid-inhibiting agents. They have also provided standards for certain studies which may throw light on such questions as to the genesis of non-toxic goiters in the presence of an adequate intake of iodine, the mode of action of iodides in Graves' disease, and as to the mechanism of action of the thyroid hormone.

Stanley and Astwood (18) have evaluated the effect of thyroid-stimulating hormone on the collection of radio iodine by the thyroids of normal humans. They determined the accumulation gradients in the thyroids of their subjects and followed

the effect of thyroid-stimulating hormone on these gradients. They observed that for eight hours after the injection of hormone no effect on the rate of iodine pickup could be detected, but thereafter a marked acceleration which became maximum in 24 to 48 hours occurred. This increased avidity for iodine observed in subjects after administering thyrotrophic hormone was not altered by administering mercaptoimidazole, an agent which effectively inhibits the binding of iodine to thyroid protein, *i. e.*, inhibits the iodination of thyroid hormone. This observation suggests that notwithstanding the mode of action of the thyroid-stimulating hormone, the increased rate of collection of iodide by the thyroid is not secondary to its being converted to thyroid hormone. Indeed it may be suggested on the basis of this observation that thyrotrophic hormone has increased the iodide space of the thyroid and that any increased thyroid hormone production following treatment with thyrotrophic hormone is secondary. They were unable to demonstrate any loss of stored iodine following the administration of thyrotrophic hormone. These results differ from those obtained by Keating *et al.* (19), who studied the effect of thyroid-stimulating hormone on the collection and release of radio iodine by the thyroids of chicks. These investigators observed a loss of iodine from the chick thyroid before any increased collection could be demonstrated. These differences might be explained as species differences or they might be related to dose levels. If one calculates the doses per unit weight of test animal, the chicks received doses 20 to 40 times greater than those received by the human subjects.

Stanley and Astwood (18) also observed that patients who were considered to have potentially normal thyroids and who were taking desiccated thyroid were incapable of accumulating significant amounts of radio iodine in their thyroids. They did observe, however, that patients who had been taking desiccated thyroid up to the time of receiving the thyrotrophic hormone were capable of concentrating radio iodine after thyroid-stimulating hormone had been administered. This inhibition to the iodine concentrating capacity of the thyroid by the administration of exogenous thyroid hormone might be attributed to an inhibition of thyrotrophic hormone production in the subject's own pituitary. It must be pointed out,

however, that Cortell and Rawson (20) have demonstrated that thyroxine when administered to hypophysectomized rats partially inhibits the action of administered thyroid-stimulating hormone on the thyroid. It would be interesting to compare the effects of thyroid-stimulating hormone on the accumulation gradients of radio iodine in the same euthyroid subjects before and after treatment with desiccated thyroid. Stanley and Astwood (18) suggest that this method of study might be of value in determining whether or not a patient being treated with desiccated thyroid does or does not have a potentially functional thyroid gland.

Studies with radioactive iodine have been of value not only in determining the mode of action of certain thyroid-inhibiting agents but such studies have also been of value in quantitating the effects of a variety of these drugs. In 1943 Rawson *et al.* (21) reported a patient who had developed a large goiter, while taking potassium thiocyanate in treatment of hypertension. Biopsy of this gland showed an extreme hyperplasia. Only a small per cent of a tracer of I^{131} given to this patient was excreted in the urine. Unfortunately this observation was not correlated with the blood thiocyanate level.

Subsequent studies by Haines (22) and by Richards (23) done in several patients having "thiocyanate goiter" have revealed that the excretion of radioactive iodine by such goiterous patients can be related to the blood thiocyanate levels observed in such patients. If the thiocyanate level is elevated, most of the iodide is excreted; if it is low, less of the iodide is excreted.

Rawson *et al.* (24) used radio iodine tracer technics in evaluating the action of thiouracil on the thyroids of Graves' disease. They prepared patients for thyroidectomy by administering thiouracil. Preoperatively they gave ten of their patients tracer doses of I^{131} and determined the radioactive iodine excreted in the urine and that recoverable in the operatively removed thyroids. They found that most of the tracer iodine was excreted in the urine and that only a small per cent was contained in the thyroids. They concluded that this drug acted to interfere with the synthesis of thyroid hormone by setting up a block to the collection or the utilization of iodide.

Studies done by Stanley and Astwood (25)

have demonstrated that in thyrotoxic humans the thiouracil group of drugs causes an inhibition to the organic bindings of iodine (which must first have been accumulated by the thyroid as iodide) to thyroid protein. By measuring with *in vivo* methods the pickup of radio iodine by the thyroid they were able to demonstrate that the thyroid, though well controlled by the administered drug, was capable of concentrating iodide in the thyroid. That this collected I^{131} existed as iodide in the well-controlled gland was evidenced by the fact that the administration of large doses of potassium iodide resulted in a sudden decrease in the activity over the thyroid. They also demonstrated that the administration of potassium thiocyanate resulted in a prompt discharge of accumulated iodide from the thyroid. It is interesting that bromide was observed by these investigators to have no effect on the collected iodide. Patients receiving thiouracil or related agents in doses inadequate to control their thyrotoxicosis when injected with potassium thiocyanate were found to discharge only a fraction of the accumulated radio iodine and were concluded to be receiving inadequate amounts of the drug to inhibit the binding of iodine to the thyroid protein. It has been suggested by Stanley and Astwood (25) that this method of study would be of value in determining the adequacy of dose in patients undergoing treatment with any of these agents. On the basis of these observations it is apparent that the first step in storing iodine in the thyroid is that of concentrating it as iodide in an iodide space or trap. It would appear from these studies that the pickup of iodide is not dependent upon the rate of iodination of the hormone. On the other hand we have observed that the pickup of iodine by the thyroids of rats under the influence of thiouracil is only a fraction of that collected by thyroids of untreated controls even at 10 minutes after administering the tracer iodine. Notwithstanding this demonstration that the iodine is first concentrated as iodide we still do not understand the mechanism by which the iodide is accumulated in the thyroid nor are we able to explain the increased avidity for iodine by the hyperplastic thyroids of Graves' disease nor by the thyroids of animals previously treated with thyrotrophic hormone.

Stanley and Astwood (26) have compared and quantitated the thyroid-inhibiting properties of

several goitrogenic agents on the thyroids of human subjects. The method that they used depended on the capacity of these agents to modify the accumulation gradients of radioactive iodine by the thyroids as detected by serial counts with an externally placed Geiger-Mueller counter. The values obtained by these investigators are considerably different from those obtained by methods which depend upon the goitrogenic effects of these drugs in rats. For example, whereas propyl thiouracil has goitrogenic properties in the rat which are 10 times those of thiouracil, by this method of assay propyl thiouracil has only 0.75 the activity of thiouracil. Likewise, thiourea, an agent possessing only 0.1 the goitrogenic activity of thiouracil in the rat is equally as effective as thiouracil in altering the accumulation gradient in human thyroids.

Rawson and McGinty (27, 28), who compared the minimal doses of various injected drugs necessary to inhibit significantly the concentration of radioactive iodine in the chick thyroid found that propyl thiouracil was no more effective than thiouracil whereas by tests of goitrogenesis in the rat propyl thiouracil was 10 times more active than thiouracil. These same investigators found that benzyl thiouracil was 10 times as effective as thiouracil in inhibiting the concentration of radioactive iodine in the thyroid of a chick. Stanley and Astwood (26) found this agent in their studies on the human to have 0.75 the activity of thiouracil. McGinty and Wilson (29) have applied the methods of Stanley and Astwood in quantitating the effects of these agents on the thyroids of the Rhesus monkey. They have found the monkey thyroid to respond like the human thyroid, both qualitatively and quantitatively to these various thyroid-inhibiting agents. On the basis of these observations we might suggest then that agents would best be quantitated on the monkey with the method described by Stanley and Astwood before being subjected to clinical trial in the human by the same method. The final test of clinical usefulness will depend of course upon testing such drugs on patients having thyrotoxicosis. The need for this final test is demonstrated by our own observations that benzyl thiouracil, which when tested in the rat and the chick seemed to be a very active agent and when tested by the method of Stanley and Astwood had about the same activity

as propyl thiouracil, was ineffective in controlling thyrotoxicosis in a small series of patients.

The mechanism by which the various salts of iodine exert their well-known therapeutic effect in Graves' disease has long remained unexplained. Indeed when one considers that the thyroid of untreated Graves' disease has an increased avidity for iodine which it rapidly converts and secretes into the circulation as thyroid hormone, the administration of iodine to such patients would seem to be an unwise maneuver. However, notwithstanding these theoretical contraindications, the therapeutic value of iodine in Graves' disease is a well-established fact. Plummer (30) who established the value of this therapeutic measure advanced the hypothesis that the toxic effects of Graves' disease were due to the elaboration of an incompletely iodinated hormone molecule. He suggested that the therapeutic action of iodine in this disease resulted from complete iodination of the hormone which even in excess was less toxic than the abnormal hormone.

In contrast to the two-hormone theory advanced by Plummer (30), Rawson *et al.* (31) have advanced a theory that iodine has two actions on the thyroid, an iodinating or a nutritive action and an involuting action on the thyroid of Graves' disease and have reported that they had been able to separate these two actions of iodine on the thyroid. They studied patients having Graves' disease before any medication was given, after treatment with thiouracil had caused a fall in the basal metabolic rate to a normal level, and after iodine had been added to the regime of treatment with thiouracil. Observations included micro-histometric studies of biopsy specimens taken before treatment, after treatment with thiouracil, but before administering iodides, and of glands removed at operation after treatment with both drugs. The urinary excretion of radioactive iodine was determined before and during treatment with thiouracil. Total and thyroglobulin iodines were determined in the operatively removed thyroids. The results showed hyperplasia of the thyroid samples removed before starting treatment with mean acinar cell heights which averaged 12.0 micra. There was an increase in thyroid hyperplasia after treatment with thiouracil, the mean acinar cell heights averaging 13.9 micra. However, involution was observed after iodine had been adminis-

tered in addition to thiouracil, the average mean acinar cell height decreasing to 7.2 micra. This involution occurred even though the thyroids were not utilizing the iodine in the synthesis of thyroid hormone as evidenced by urinary excretions of radio iodine during treatment with thiouracil which averaged 80 per cent as contrasted to average radio iodine excretions of 25 per cent before beginning treatment. The failure of such glands to utilize iodine in the synthesis of thyroid hormone is further supported by the thyroglobulin iodine values which in the glands taken from patients who had received thiouracil and iodine averaged 7.0 mgm./100 gm. of fresh thyroid tissue. The average thyroglobulin iodine of a comparable series of patients who had been prepared for operation with thiouracil alone was 6.8 mgm./

100 gm. of fresh thyroid tissue, of normal human thyroids 29.8 mgm. per cent, of a small series of patients treated with iodine only, 36.6 mgm. per cent.

All of the iodine administered to one patient was labelled with radioactive iodine and thus a balance study was made. Before treatment only 16.3 per cent of a tracer dose was excreted. After treatment with thiouracil 0.8 gm. daily for 15 days, 73.5 per cent of an identical dose of radio iodine was excreted. Following the second biopsy taken from the thyroid, a daily dose of 300 mgm. of sodium iodide labelled with 100 microcuries of radioactive iodine was administered in addition to thiouracil 0.8 gm. daily. The radioactivity demonstrated in the urine excreted during and after the 10 day period of treatment with thiouracil and

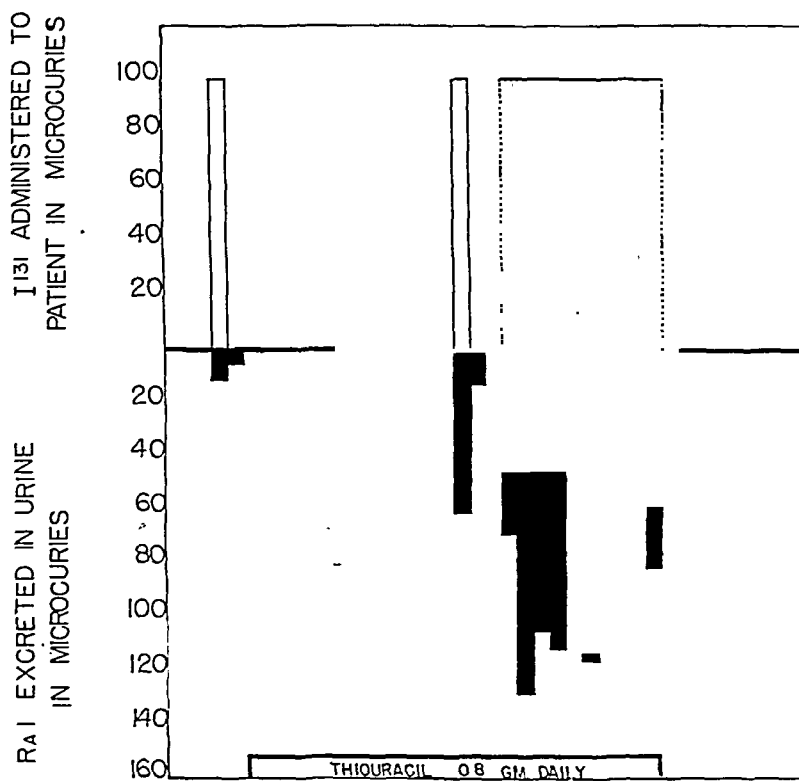


FIG. 1. RADIO IODINE BALANCE IN PATIENT BEFORE TREATMENT WITH THIOURACIL, DURING TREATMENT WITH THIOURACIL AND DURING TREATMENT WITH THIOURACIL AND SODIUM IODIDE

Intake above the line. Urinary excretion below. With regard to intake plain white columns indicate that tracer iodine was given with a carrier of 150 gamma of non-radioactive sodium iodide. Stippled columns indicate that radioactive iodine was given as tracer for therapeutic dose of 300 mgm. daily of ordinary sodium iodide. (From Rawson, Moore, Peacock, Means, Cope and Riddell, *J. Clin. Invest.*, 1945, 24, 875.)

labelled sodium iodide practically equalled the radioactivity administered. See Figure 1. The radioactivity demonstrated in this patient's operatively removed thyroid was too little to measure and the thyroglobulin iodine value was only 2.3 mgm. per cent. Notwithstanding the fact that this gland failed to accumulate or to convert the administered iodine to thyroglobulin, the thyroid tissue examined after treatment with sodium iodide was well involuted in contrast to an extreme hyperplasia observed in the biopsy specimen taken just before adding iodine to the treatment. See Figure 2. This separation of the involuting action of iodine from its nutritive action, these investigators believe, may explain the paradoxical effect of iodine in Graves' disease. They also suggested that the involuting effect of iodine in Graves' disease was due to an inhibitory action of this agent to the action of thyrotrophic hormone on the thyroid cell.

Space will not permit further discussion as to the mechanism of this involuting action of iodine on the thyroid. However, attention should be

called to the reports of Wolff and Chaikoff (32), who by using tracer techniques have demonstrated in rats that the maintenance of an inorganic blood iodide level of 200 μ g. per cent or more interferes with the organic binding of iodine by the thyroid. They suggest that the level of plasma iodide is part of a homeostatic mechanism which governs hormone synthesis in the normal gland.

Although there is good evidence that endemic goiter is due to a deficiency of iodine intake, it is difficult to explain the development of such non-toxic goiters in certain patients who apparently have an adequate intake of iodine. Many students of thyroid disease have suspected the existence of positive goitrogens which play a role in the development of some of the non-toxic goiters developing in subjects whose iodine intakes by accepted standards are more than adequate. Greer and Astwood (33) have demonstrated that certain foods have an inhibitory effect to the accumulation of radio iodine. They have evaluated the effects of 61 different food substances on the "accumulation gradient" of radio iodine in normal hu-

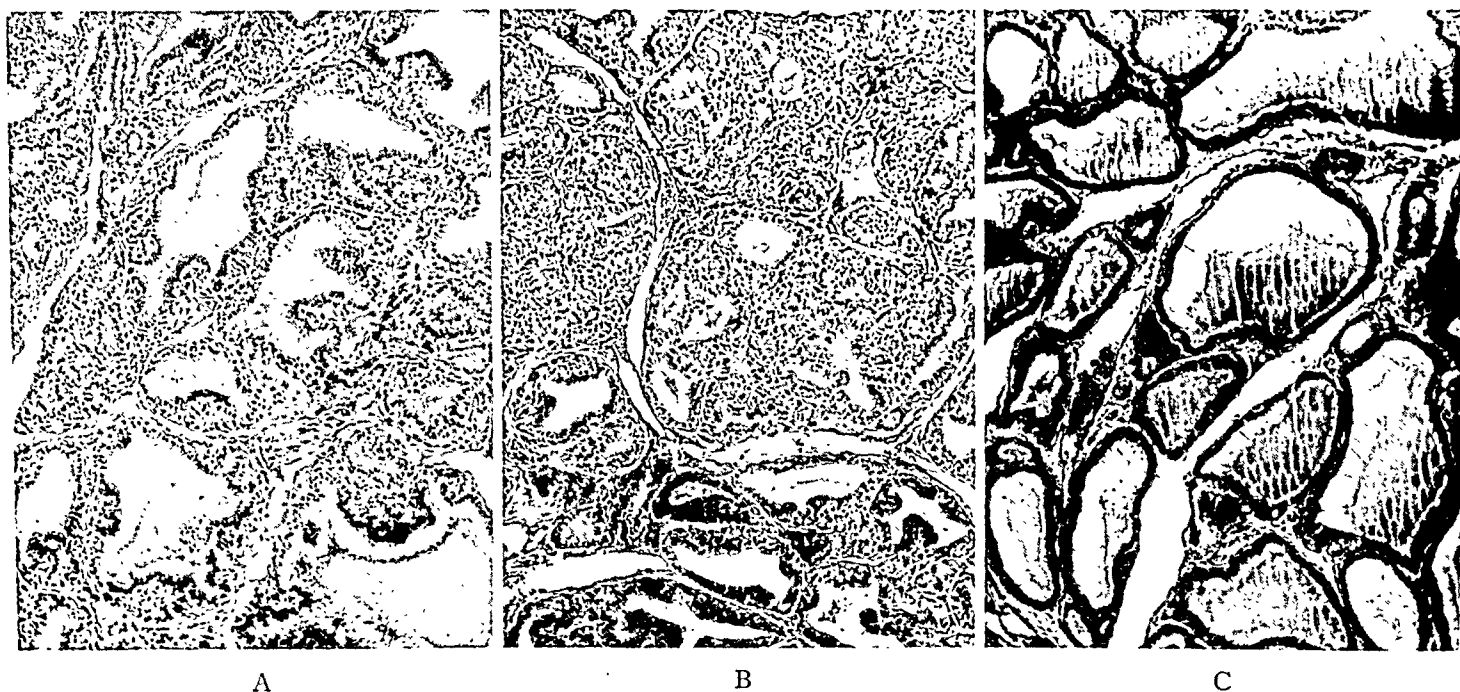


FIG. 2. HISTOLOGIC SECTIONS TAKEN FROM BIOPSY SPECIMENS AND OPERATIVELY REMOVED THYROID IN PATIENT TREATED WITH THIOURACIL AND THIOURACIL PLUS SODIUM IODIDE

A. Histologic section of biopsy specimen taken before any treatment. Mean cell height 15.3 ± 0.14 .

B. Histologic section of biopsy removed after treatment with thiouracil had caused a fall in the B.M.R. to a euthyroid level. Mean cell height 17.3 ± 0.17 .

C. Histologic section of operatively removed thyroid after treatment with thiouracil and iodide. Mean cell height 10.3 ± 0.07 .

(From Rawson, Moore, Peacock, Means, Cope and Riddell, *J. Clin. Invest.*, 1945, 24, 873.)

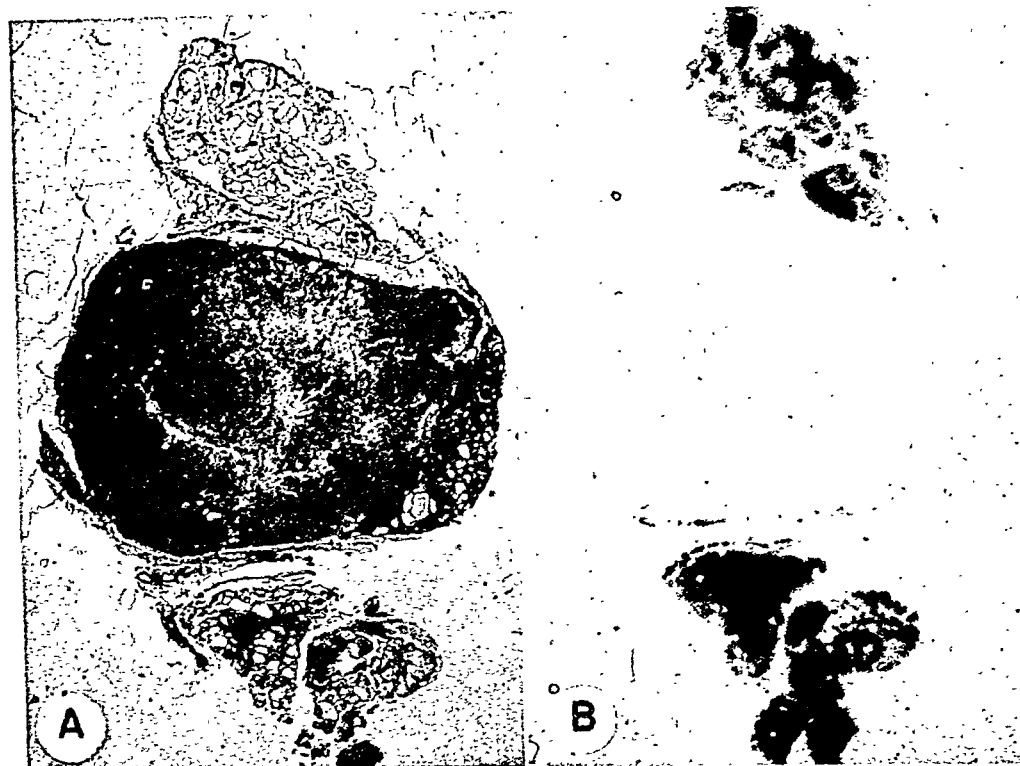


FIG. 3. A SOLID CELLULAR OR TRABECULAR ADENOMA SURROUNDED BY NORMAL THYROID TISSUE WITH AUTORADIOGRAM

A. Histologic section.

B. Autoradiogram showing no evidence of function in the adenoma. The areas of black coincide with the normal thyroid tissue.

(From Dobyns and Lennon, *J. Clin. Endocrinol.*, 1948, 8, 735.)

man thyroids, and have observed that the more active foods in inhibiting the thyroid's accumulation of radioactive iodine are rutabaga, turnips, string beans, frozen peaches, pears, peanuts, walnuts, liver, milk and oysters. On the basis of these studies we might suggest that the sporadic goiters seen in areas where the iodine intake is adequate are related to positive goitrogens contained in certain dietary products.

Radioactive iodine has been a most valuable tool in evaluating the function of certain tumors of the thyroid. Indeed studies with radioactive iodine have stimulated an interest in neoplasms of the thyroid, benign and malignant, that has probably never existed before. The benign tumors of the thyroid have been studied extensively by Cope *et al.* (34), Rawson *et al.* (35), and by Dobyns and Lennon (36). In general it can be said that the benign tumors have an avidity for radio iodine which can be correlated with the degree of histologic differentiation. The solid cellular tumors,

trabecular and tubular adenomas, which show no differentiation have been found to manifest practically no iodine concentrating capacity whereas the microfollicular adenomas which show definite but incomplete differentiation have been observed to have an avidity for iodine approaching that of normal thyroid tissue. See Figures 3 and 4. At the other end of the spectrum single adenomas have been found in patients having hyperthyroidism which was relieved by simple removal of the adenoma plus a biopsy specimen of uninvolved tissue. In these instances the radio iodine was found concentrated in the adenoma and none in the uninvolved tissue. Such tumors presented histologic pictures of hypertrophied microfollicular adenomas or frank hyperplasia. The surrounding uninvolved tissue showed hyperinvolution or decreased acinar cell heights.

Dobyns and Lennon (36) attempted to correlate the cell heights of these adenomas with their capacities to concentrate radio iodine. They found

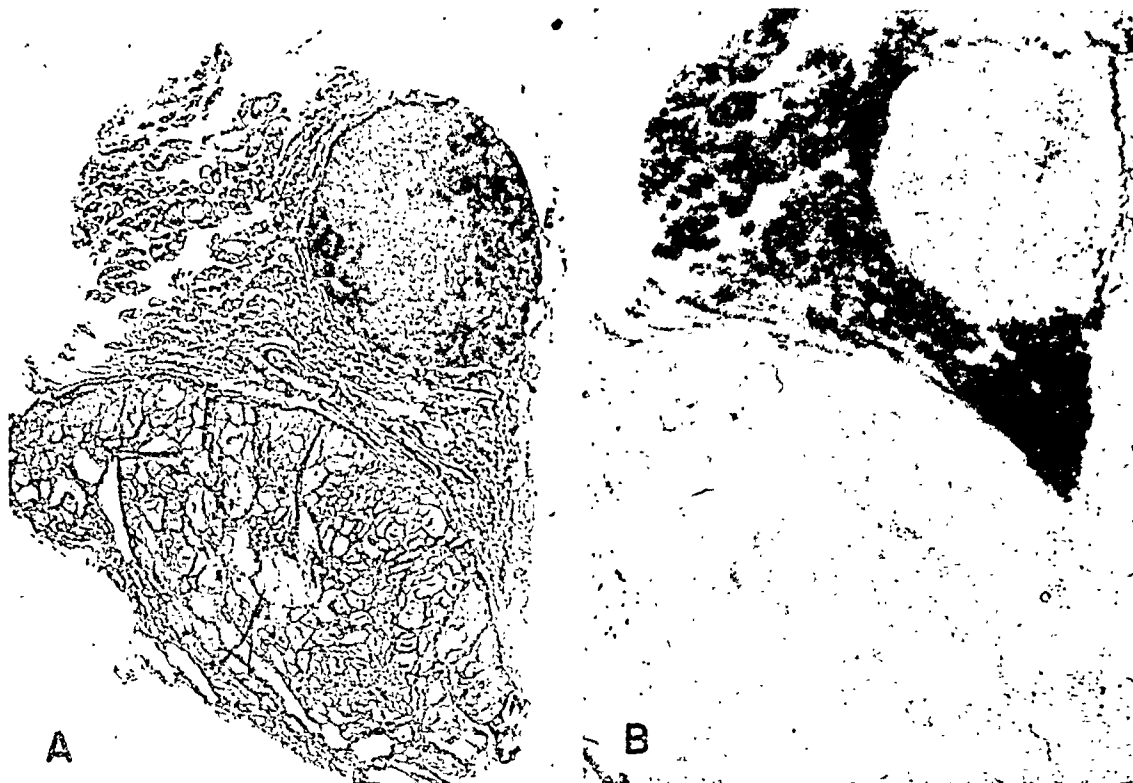


FIG. 4. TWO FUNCTIONAL ADENOMAS OF THE THYROID SEPARATED BY NORMAL THYROID TISSUE

A. Histologic section. Adenoma above is tubular and microfollicular; below is a micro and macrofollicular adenoma which has certain characteristics suggestive of a colloid adenoma.

B. Autoradiogram in which both adenomas were observed to have low-grade iodine concentrating capacity, but much less than that observed in the areas corresponding to the normal thyroid tissue between the two adenomas.

(From Dobyns and Lennon, *J. Clin. Endocrinol.*, 1948, 8, 738.)

a certain group of hyperplastic adenomas which did not concentrate radio iodine. Such tumors showed a marked variability in their cell heights and as such resembled a group of papillary adenocarcinomas. See Figure 5. The tumors which were functioning in excess of the surrounding thyroid tissue were found to have mean cell heights significantly and uniformly in excess of the surrounding normal thyroid tissue. See Figure 6. They not only found hyperfunctioning adenomas as evidenced by an increased pickup of radio iodine, which were associated with clinical hyperthyroidism, but they also found certain small adenomas which concentrated more radio iodine and had uniformly increased cell heights as compared to the surrounding tissue in patients who had no elevation in the basal metabolic rates. Continuation of such studies may throw considerable light on the life history of hyperfunctioning adenomas of the thyroid as well as upon the history of certain papillary adenocarcinomas.

Studies of cancer of the thyroid with radioactive iodine have not only excited an unprecedented interest in this disease, but they have already resulted in a much better understanding of the biology of this type of cancer. Furthermore, this writer believes these studies have already led to improvements in treatment of this disease with the conventional forms of therapy. Hamilton *et al.* (37) were the first investigators to apply radio iodine methods in studying cancer of the thyroid. With autoradiographic technics they demonstrated that normal thyroid tissue concentrated labelled iodine in the thyroid follicle. With this same technic they studied two cancers of the thyroid and observed that the cancerous tissue was incapable of concentrating any radio iodine. The first successful attempt to demonstrate pickup of radioactive iodine by thyroid cancer was reported in 1942 and 1944 by Keston *et al.* (38) and by Frantz *et al.* (39). They administered tracer doses of radio iodine to three patients having thy-

roid tumors with bony metastases. One bony metastasis from one of these tumors, an adenoma malignum, showed an appreciable uptake of radio iodine.

In 1946, Leiter *et al.* (40) reported two cases of adenocarcinoma with functioning metastases and hyperthyroidism which they studied with radioactive iodine. One of these patients had been totally thyroidectomized in 1923. Tracer studies with radioactive iodine done in this patient demonstrated that there was no functioning thyroid tissue in the neck and that the source of hyperthy-

roidism was in the metastatic tumors. Seidlin *et al.* (41) have reported that treatment of this patient with radioactive iodine resulted in definite and lasting improvement.

Following these observations several investigators interested in using radio iodine in the therapy of cancer of the thyroid have undertaken studies with radio iodine concerning the natural avidity of various thyroid cancers for radio iodine and means of increasing the capacity of such tumors to concentrate radioactive iodine.

Marinelli *et al.* (42) have investigated with

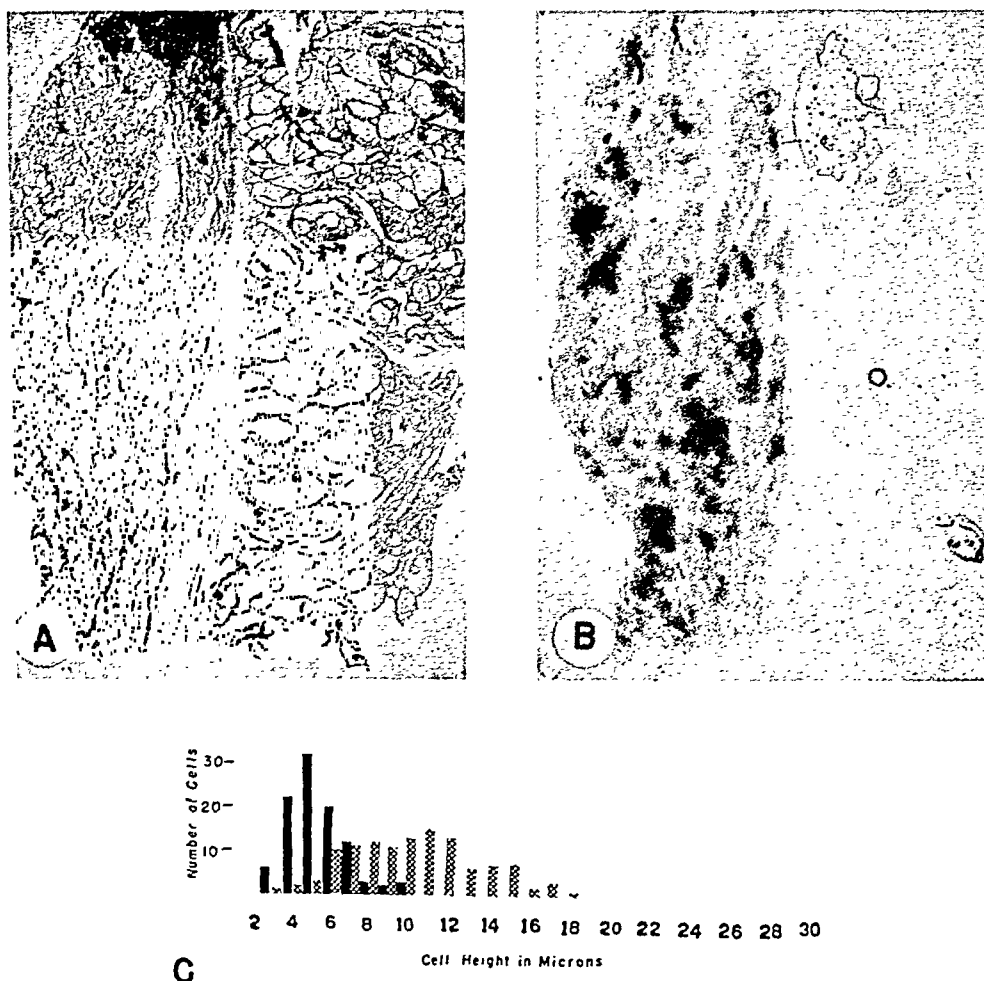


FIG. 5. HYPERPLASTIC ADENOMA WITHOUT ANY IODINE CONCENTRATING FUNCTION

A. Histologic section. Adenoma on right; extranodular tissue on left.

B. Autoradiogram showing that the radio iodine is concentrated in the extranodular tissue rather than in the adenoma.

C. Cell heights (100 cells in each type of tissue). Black columns represent the cells in the extranodular tissue; checkered cells represent the cells in the adenoma, showing an increased mean cell height but with considerable variability.

(From Dobyns and Lennon, *J. Clin. Endocrinol.*, 1948, 8, 739.)

radioautographic technics the capacity of certain cancers of the thyroid to accumulate radioactive iodine. They studied 19 selected cancers of the thyroid and observed that 10 of these tumors possessed the ability to accumulate radioactive iodine. Five of their functioning tumors presented the histologic structure of so-called "benign metastasizing struma." See Figure 7. The remaining five tu-

mors which exhibited this evidence of function had the structure of follicular adenocarcinoma in some portion of the material studied. They concluded that pickup of iodine is closely linked with certain structural qualities, which include orderly cell arrangement in follicular pattern and the presence of colloid-like material. See Figure 8. Frantz *et al.* (43) in a study done on 32 cancers

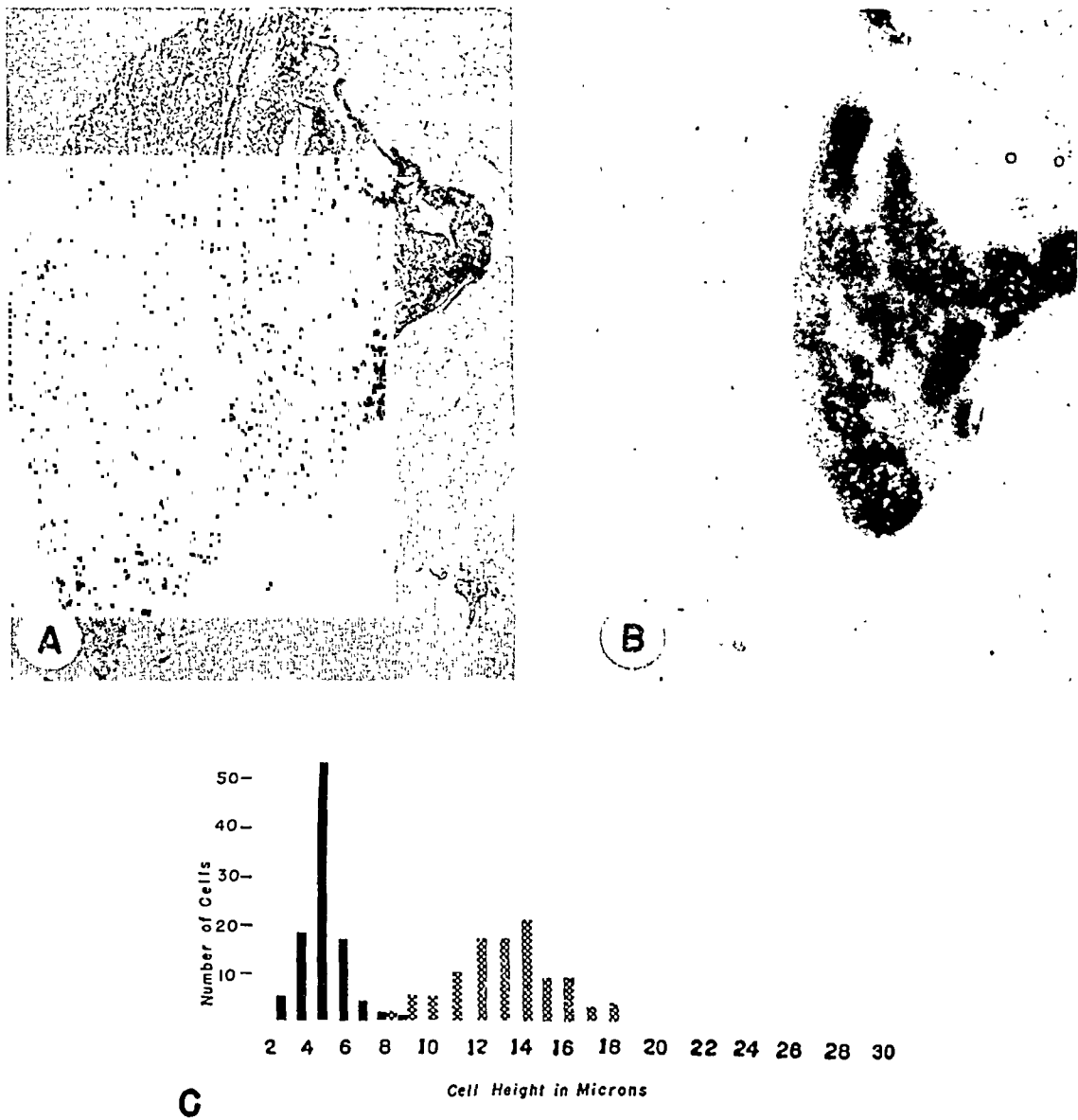


FIG. 6. HYPERPLASTIC ADENOMA WITH HYPERFUNCTION

- A. Histologic section. A roughly triangular piece of adenomatous tissue is shown on the right; uninvolved tissue on the left.
- B. Autoradiogram showing the relatively high concentration of radio iodine in the adenoma and practically none in the remaining thyroid tissue. The adenoma is interpreted as being an adenoma functioning in excess of the remaining tissue.
- C. Cell heights (100 cells in each type of tissue). Black columns represent the cells in uninvolved tissue; checkered columns represent cells of adenoma and illustrate the relative uniform cellular hypertrophy of hyperfunctioning thyroid adenomas.
- (From Dobyns and Lennon, *J. Clin. Endocrinol.*, 1948, 8, 737.)

of the thyroid observed that seven out of 12 malignant adenomas and six mixed tumors picked up radio iodine. No pure papillary adenocarcinomas nor less differentiated cancers of the thyroid picked up the isotope.

Fitzgerald (44) reported before the Brookhaven conference that in a study of 47 selected cases of carcinoma of the thyroid given tracers of I^{131} prior to surgical removal, 20 cases showed retention of the radio iodine in some portion of the neoplastic areas. He reported that pure papillary adenocarcinomas of the thyroid did not retain the iodine. In five out of eight mixed papillary and follicular or alveolar adenocarcinomas, a retention of radio iodine was observed. He reported that two-thirds of the follicular and alveolar carcinomas stored radio iodine. Two cases of solid alveolar carcinoma stored the radioactive iodine. In six cases of Hurthle cell carcinoma and in two cases of

spindle and giant cell carcinoma he found no storage of radio iodine.

Seidlin *et al.* (45) reported that eight out of 14 patients having cancer of the thyroid were capable of collecting radio iodine. They reported that the destruction of normal thyroid tissue by radioactive iodine resulted in an increased avidity for iodine by the cancer tissue in two of three patients. They also reported that one out of two patients treated with thyroid-stimulating hormone showed an increased pickup of iodine.

Rawson *et al.* (46) have observed the effect of thyroidectomy, surgical or by large doses of radio iodine, in 21 patients having relative or absolute non-functioning metastatic thyroid cancer. Following removal or destruction of the normal thyroid eight solid and/or alveolar adenocarcinomas assumed the capacity to concentrate radioactive iodine.

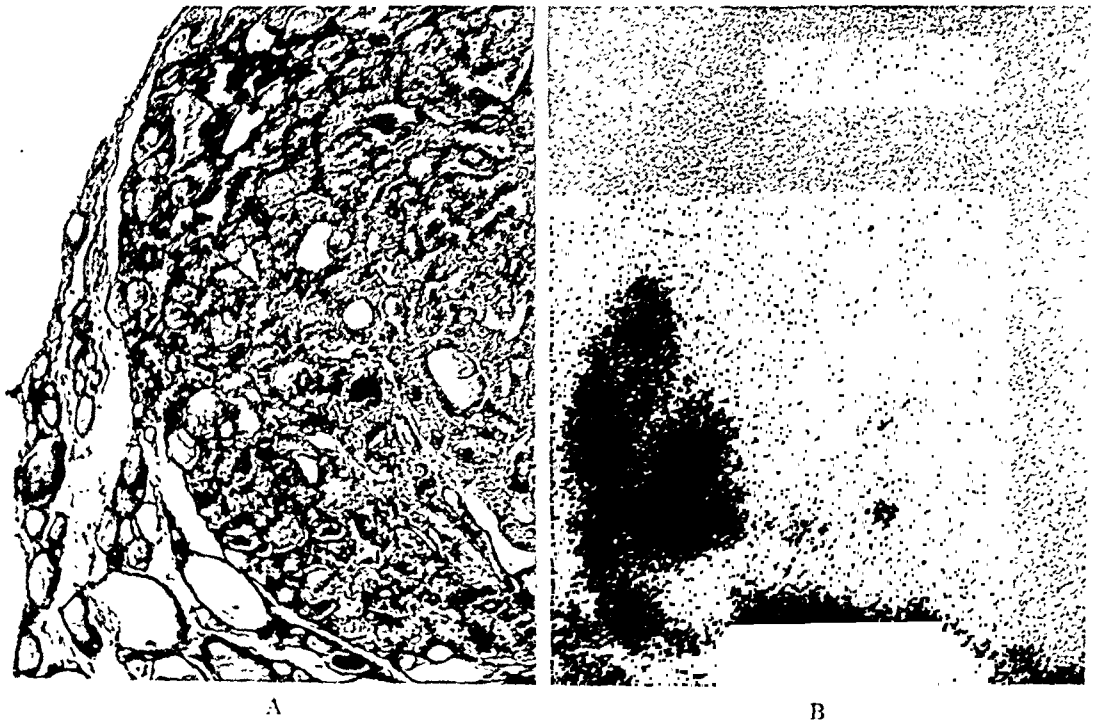


FIG. 7. MICROFOLLICULAR ADENOCARCINOMA OF THE THYROID SURROUNDED BY A FEW APPARENTLY NORMAL THYROID FOLLICLES

A. Histologic section.

B. Autoradiogram made by applying this section on photographic film. It would be noted that the maximum function is demonstrated in the uninvolved normal thyroid tissue. It will also be noted that the tumorous tissue concentrated significant amounts of radioactive iodine, but much less than that concentrated by the normal thyroid tissue.

(From Marinelli, Foote, Hill, and Hocker, *Am. J. Roent.*, 1947, 58, 26.)

A case history of the first patient in whom they tried this experiment is quoted from their report:

"Mrs. T. I., M. G. H., number 48114, aged 43; was the first patient in whom this experiment was tried. She was admitted to the Massachusetts General Hospital on February 28, 1945, complaining of a soft tissue mass in the skull and of regrowth of a goiter. She gave a history of having had surgical removal in 1933 of a thyroid nodule which had existed for five years. She reported that one year before admission to this clinic she had first noted a lump over the occiput, and that six months before admission she had noted an extensive regrowth of her goiter. She was found to have a lemon-sized, moderately firm mass in the left occipitoparietal area measuring about 5×6 cm. in diameter and raised about 2 cm. above the rest of the skull. She was also found to have a walnut-sized, hard mass in the upper pole of the right lobe of the thyroid. Several large firm lymph nodes were palpable in the right anterior cervical chain. Clinically she was euthyroid and her basal metabolic rate was minus 12 per cent. X-ray studies of the chest and skeleton revealed only an osteolytic defect in the left parietal bone consistent with destruction by metastatic disease.

Tracer studies done with radioactive iodine revealed that the occipital metastasis was capable of concentrating only minimal amounts of iodine. It was reasoned that removal of her normal thyroid might be followed by a significant pickup of iodine by the skull metastasis. On March 6, 1945, Dr. R. R. Linton did a total thyroidectomy and a right radical neck dissection. At the same time a biopsy specimen was removed from the metastatic lesion in the skull. The thyroid showed a follicular adenocarcinoma. A similar picture was found in the small metastasis though this lesion showed more differentiation than the primary lesion and contained some colloid filled acini. Her normal thyroid was found to have collected 1.8 per cent of the preoperatively administered radioactive iodine per gram of tissue. The skull metastasis was found to have collected only 0.06 per cent of administered radio iodine per gram of tissue. On March 27, another tracer dose of radioactive iodine was administered and *in vivo* measurements over the metastatic lesion were made. Though the patient was found to concentrate less than 3 per cent of administered iodine in the skull metastasis, she did not develop myxedema in a period of four months. Indeed, in July 1945, her basal metabolic rate was plus 4 per cent. Hav-

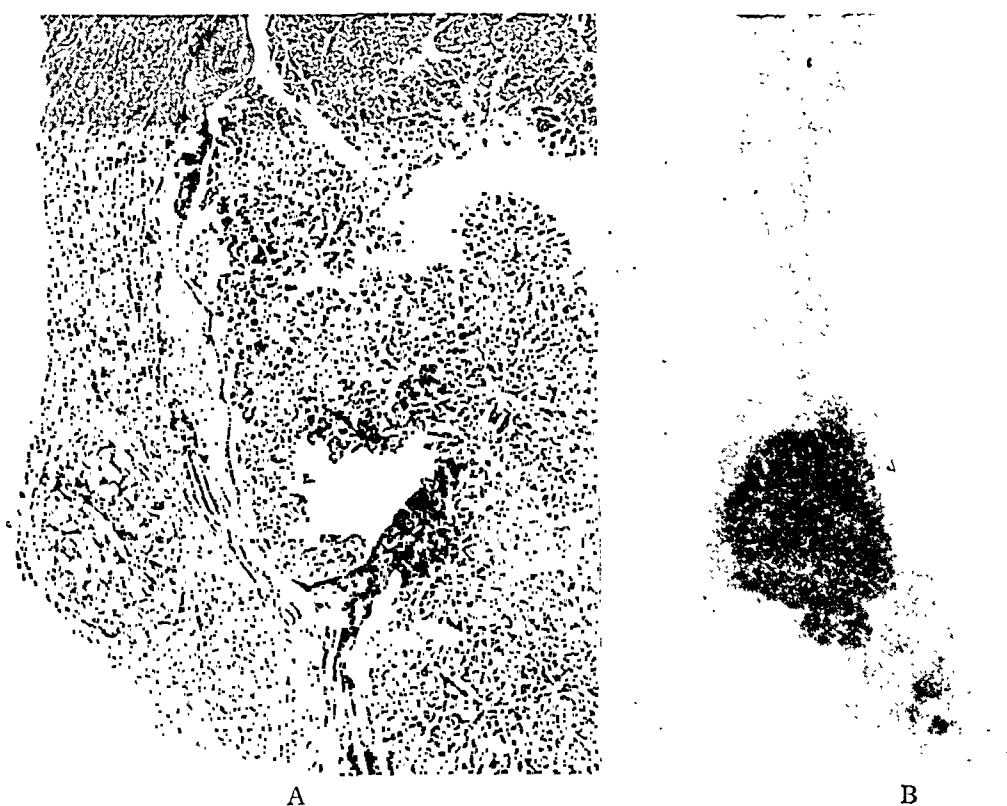


FIG. 8. PAPILLARY CARCINOMA OF THE THYROID SURROUNDED BY UNINVOLVED THYROID TISSUE
A. Histologic section of papillary carcinoma of the thyroid surrounded by uninvolved thyroid tissue and encapsulated micro et microfollicular adenoma.

B. Autoradiogram made by placing Section A on photographic film. It is to be noted that the cancerous tissue in this instance did not concentrate any radio iodine. It is of interest that the benign lesion in the extrathyroidal tissue is functional and indeed concentrated more iodine than did the surrounding uninvolved thyroid tissue.

(From Marinelli, Foote, Hill, and Hocker, Am. J. Roent., 1947, 58, 23.)

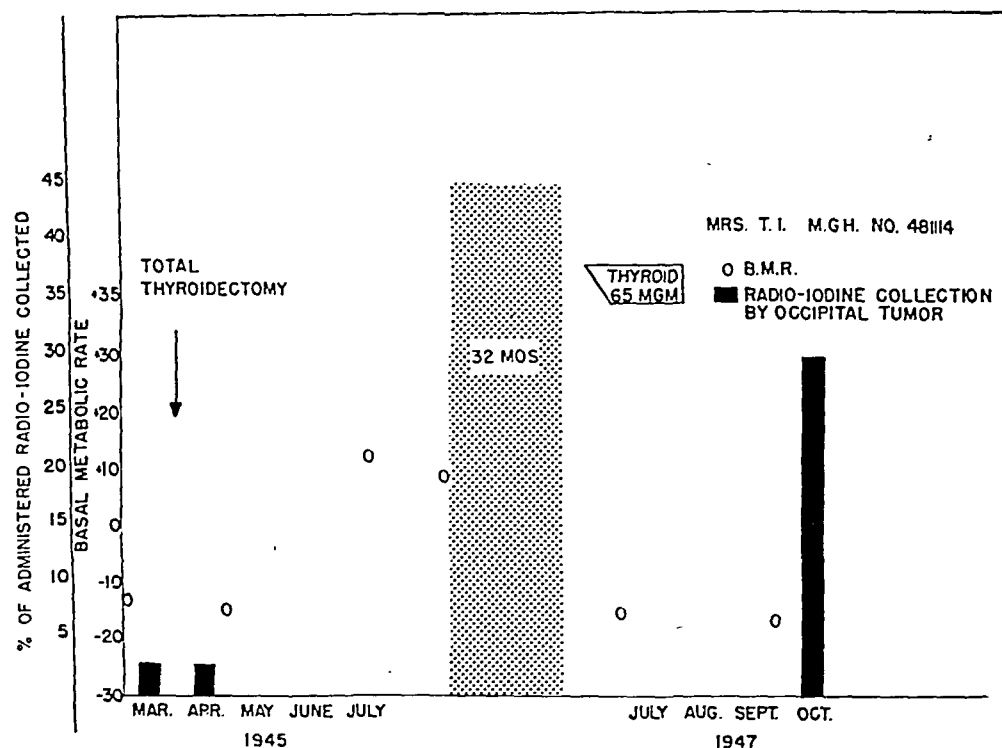


FIG. 9. B.M.R. LEVELS AND RADIO IODINE COLLECTIONS BY METASTATIC TUMORS BEFORE AND AFTER REMOVAL OF THE NORMAL THYROID

It is to be noted that this patient concentrated considerably more radioactive iodine in her metastatic tumor after removal of the normal thyroid than she did before operation. It is also to be noted that four months after total removal of the normal thyroid her B.M.R. was within normal range. (From Rawson, Marinelli, Skanse, Trunnell and Fluharty, J. Clin. Endocrinol., 1948, 8, 832.)

ing the erroneous impression that thyroidectomy was ineffective in altering the function of this tumor, it was decided to attempt removal of the skull metastasis. On July 6, Dr. Mixter made this attempt. However, it was impossible to do more than a partial removal of the tumor. Postoperatively the patient was treated with x-ray and received 1550 roentgens delivered in six doses through a 10×10 field. The patient returned to her home in Rochester, New York, and was not seen again until July of 1947. At that time she was taking desiccated thyroid in a daily dose of 65 mgm. (1 grain). She reported that in April of 1947 she had noted a regrowth of the tumor in her occipitoparietal area. She was found to have a moderately firm tumor, which measured about 2 cm. in diameter, at the site of the previous occipital tumor. She had a basal metabolic rate of minus 16 per cent. A tracer dose of radioactive iodine was given on July 3, of which she excreted 92 per cent in the urine. *In vivo* measurements revealed radioactivity in the skull lesion and in the neck. There was about three times as much activity in the skull metastasis as in the neck. On August 1, she was advised to stop taking thyroid. She was readmitted to the hospital in October of 1947 and was found to have a B.M.R. of minus 11 per cent. Studies revealed an increase in

size of the occipital lesion and a new osteolytic lesion in the third dorsal vertebral body. A tracer dose of radioactive iodine was administered on October 6. Of this tracer dose, she excreted only 48 per cent. By *in vivo* measurements, it was estimated that she concentrated 30 per cent of the administered dose in her occipital lesion, 10 per cent in the region of her thyroid and 10 per cent in the vertebral lesion. This patient is now being treated with large doses of radioactive iodine." See Figure 9.

The time required to observe these changes subsequent to thyroidectomy varied between one month and 32 months. In four cases of pure papillary adenocarcinoma that they studied, total thyroidectomy had no effect on the function of previously non-functioning metastasis. These same investigators have likewise reported that the avidity for radio iodine of certain functioning cancers of the thyroid can be increased by administering thyroid-stimulating hormone. They have also observed that prolonged treatment with thiouracil may be followed by considerable increase in the

iodine accumulating capacity of certain functioning cancers of the thyroid. This described effect of prolonged treatment with thiouracil might be ascribed to an increased elaboration and augmentation in action of the patient's own thyroid-stimulating hormone.

These observations in which it has been demonstrated that the function of metastatic cancer of the thyroid can be altered by removal of the normal thyroid or by administering thyroid-stimulating hormone suggest that this cancer is not wholly autonomous but is capable of responding to certain physiologic stimuli. They also provide us with concepts which may lead to a more complete understanding of the biology of this type of a neoplasm. It is hoped that these concepts may also be applied in studying neoplasms of other tissues as well.

In the light of present knowledge, our understanding as to the mechanisms through which the thyroid hormone exerts its actions on the cells of the body is a void. Recent studies, however, indicate that tracing labelled thyroid hormone through its circuit may throw some light on the mode of action of this hormone. Gross and LeBlond (47) have observed that thyroxine labelled with I^{131} when administered to intact rats is withdrawn from the blood stream and is demonstrable in various tissues within two hours of its administration. They also observed that it is broken down within the body to diiodotyrosine and iodide. Hamilton, 1948 (48) and Keating and Albert (49) have observed the fate of radio iodine labelled thyroxine, thyroglobulin and iodinated casein in myxedematous patients. They have observed that less than 5 per cent of the labelled iodine is excreted in the feces and that the rest is excreted in the urine. They have found that more than 90 per cent of the labelled iodine excreted in the urine is excreted as iodide and that the remainder is excreted as diiodotyrosine. One might suggest on the basis of these studies that the thyroid hormone in the process of exerting its action is broken down and the iodine liberated to be excreted or utilized in the synthesis of thyroid hormone. It is to be hoped that these studies are only the beginning of investigations which may elucidate the mode of action of the thyroid hormone.

SUMMARY

1. Studies with radioactive iodine have resulted in major contributions to our knowledge of thyroid physiology, normal and morbid.

2. Thus far the completed studies are in harmony with previous observations made with the conventional biochemical technics. However, because of the fact that with these technics, physiologic levels of study never before obtained are possible, we have been able to broaden our knowledge of thyroid disease at a level which could not otherwise be reached.

3. With these technics we have been provided with better concepts as to

- a. the actions of certain thyroid stimulators and inhibitors,
- b. the function and biology of benign and malignant neoplasms of the thyroid,
- c. a possible mode of action of the thyroid hormone on the tissues of the body.

4. It is suggested that these studies are only the beginning of new and intensive investigations on normal and morbid physiology of the thyroid which will not only lead to better understanding of thyroid disease but may also result in more intelligent and physiologic therapy of these maladies.

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THE USE OF RADIOIODINE IN PHYSIOLOGICAL AND CLINICAL STUDIES ON THE THYROID GLAND

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The presence of iodine within the molecule of thyroid hormone has greatly facilitated physiological studies on the thyroid gland. In a sense the primary function of the thyroid gland is to regulate the intermediary metabolism of iodine; studies on the fate of iodine in the body, then, bear directly on thyroid physiology. Chemical methods for the detection and quantitative determination of iodine have been developed to a high degree of sensitivity and accuracy, and there are few substances that can be measured by chemical means in such small quantities. The use of radioiodine has not only simplified many procedures which would be most difficult by chemical methods, but it has made it possible to carry out studies which could not have been done by any other means.

First described by Fermi in 1934 (1) radioiodine (I^{128}) was first used in physiological studies by Hertz, Roberts, and Evans in 1938 (2). The longer lived isotopes, I^{130} and I^{131} , more suitable for physiological and clinical experiments, could be produced in limited quantity in the cyclotron and were used in several centers for fundamental investigations. Extensive applications of radioiodine have been possible since 1946 when I^{131} was made in quantity by the chain-reacting uranium pile.

Evidence for physiological identity of radioiodine and I^{127}

By analogy with the isotopes of other elements it was to be expected that radioactive iodine would not differ chemically or in physiological reaction from ordinary iodine. No single experiment with radioiodine establishes this fact, but many observations are consistent with the chemical identity of the several isotopes; no evidence has yet been brought forward to indicate that chemical differences exist. Favoring the supposition that

I^{131} is identical with I^{127} in chemical and physiological reactions are the observations that: (1) under appropriate conditions exchanges to theoretical equilibrium can be observed between radioiodide and diiodotyrosine and vice versa (3); (2) various radioiodine-labelled compounds can be purified to constant specific activity; (3) when physiological experiments are performed using radioactive and chemical methods for iodine in parallel, good agreement is observed; and (4) administered radioactive iodine appears in diiodotyrosine, thyroxine, and protein-bound iodine.

A question which seems not to have been answered, however, concerns the chemical state of the carrier-free iodine which is used in tracer studies. One wonders whether the equilibria which obtain with chemically detectable concentrations of iodine still hold in solutions of almost infinite dilution, and whether one can safely manipulate solutions of carrier-free I^{131} without fear of unpredictable changes in chemical state. The preparation of this material for administration to man or animal must surely carry the hazard of a partial conversion to hypoiodite or even iodate; and, were this to occur, bizarre results might follow, especially after parenteral use. For these and other reasons the use of carrier would seem advisable whenever there is no contraindication to it.

Similar uncertainties may arise when tissues or body fluids containing radioiodine are subjected to chemical procedures. When carrier cannot be used as in the identification of a new or unknown compound, large errors might enter because of the extreme smallness of the quantities involved.

Considerable debate has concerned the extent to which exchange reactions might complicate physiological studies and thus invalidate conclusions. Leblond and Süe (4) found little or no exchange *in vitro* upon admixing radioiodine and iodate or radioiodine and diiodotyrosine in aqueous solution. Miller *et al.* (3), however,

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have defined certain *in vitro* conditions which are favorable for the exchange between the iodine atoms of diiodotyrosine and iodide or iodine. The reaction is rapid in acid solutions and is accelerated by heat and by oxidative environments which would permit the presence of iodine. At physiological hydrogen ion concentrations, little exchange occurs, and reducing agents are strongly inhibitory. Evidence against the occurrence of simple exchange *in vivo*, as well as against it taking place during the usual *in vitro* fractionation procedures on thyroid tissue, has been provided by the experiments of Morton and Chaikoff (5). They showed that although radioiodine was incorporated into diiodotyrosine and thyroxine by surviving thyroid slices, little or no radioiodine entered these compounds when homogenized thyroid tissue was used.

Absorption, distribution, and excretion of iodide ion

It is usually assumed that iodide ion resembles chloride ion in the rate and extent of its absorption from the intestinal tract and in its distribution in the body fluids. It appears to remain in the extracellular space and to be distributed in the body in a manner which is very similar to chloride and thiocyanate (6). Apparently iodide ion is handled somewhat differently from chloride by the gastric mucosa and the kidney, and, of course, the difference is striking in the case of the thyroid gland. Davenport (7) found that iodide is selectively excreted by the stomach provided the blood concentration is low. As the normal concentration is very low, one would have anticipated that tracer doses of I^{131} would be selectively excreted into the stomach. This has proved to be the case (8), and it is a common observation that shortly after the administration of radioiodine a significantly higher concentration is detectable in the stomach than in other sites, excepting the thyroid and the urine. Marinelli and Hill (9) have calculated that when large doses of I^{131} are given for the treatment of thyroid tumors, the gastric mucosa receives a large dosage of radiation, even when the radioiodine is given intravenously. A temporary decrease in both gastric and salivary secretion has been noted under these circumstances (10). The kidney, at least in some species, excretes iodide

more rapidly than chloride or bromide, and the rate of excretion has been observed to be hastened somewhat by the administration of increased amounts of chloride. This has been strikingly demonstrated recently in the dog. Riggs (11) observed that this species excreted iodide very slowly but that the rate of excretion was greatly enhanced by large amounts of either bromide or chloride, and even by large amounts of iodide.

The renal excretion of a tracer dose of I^{131} in man has been extensively investigated. When a large quantity of iodide labelled with radioiodine is given, virtually all of it can be recovered in the urine within a few days. With small doses or tracer amounts only a fraction appears in the urine, a varying proportion entering the thyroid in firm combination with protein. Hamilton and Soley (12) found that 53 to 81 per cent of a 14 mg. dose was excreted in the urine by normal persons in 24 hours, while a larger fraction was excreted at a somewhat slower rate by individuals with myxedema. Hertz, Roberts, and Salter (13) found that less radioiodine appeared in the urine in cases of hyperthyroidism, a larger proportion being held in the thyroid gland. Again large doses of carrier favored the excretion of a larger proportion of the administered radioiodine. When 2 mg. of carrier or less was used in 11 thyrotoxic individuals, only 10 to 56 per cent of the dose could be recovered in the urine. It developed, therefore, that the proportion of a small dose or a tracer dose of iodine excreted in the urine bore an inverse relation to that taken into the thyroid gland. Several workers have used this phenomenon to study thyroid function in man in preference to direct measurements on the thyroid gland (see below).

Iodide ion can also be detected in other body secretions including saliva, tears, sweat, and milk, and it enters serous effusions and the cerebrospinal fluid (14). Radioiodine has not been used extensively to check these observations, but it can readily be detected in the saliva after it is given by mouth or by injection (8), and it has been found to enter the cerebrospinal fluid slowly.

Concentration of iodide ion by the thyroid

Earlier work with I^{127} showed clearly that the thyroid is capable of accumulating iodine at a rapid rate and that this iodine is quickly bound

to protein (15). Tracer studies with radioiodine have established that two steps are involved in this process: (1) The accumulation of iodide ion, and (2) the organic binding of iodine. It has long been known that a small amount of inorganic iodide can be detected in the normal thyroid gland and that considerable amounts may be found after iodine therapy. Large amounts of inorganic iodide are concentrated in the thyroid in hyperthyroidism after the administration of therapeutic quantities of potassium iodide (16). Lein (17) demonstrated in rabbits, given 35 μ g. of iodide labelled with radioiodine, a prompt accumulation of inorganic iodide followed by a slow accumulation of iodine in a protein-bound form. A still clearer demonstration of the two separate mechanisms involved in iodine collection by the thyroid was given by Franklin and Chaikoff in 1944 (18). It was observed that while sulfonamides inhibited the incorporation of I^{131} into diiodotyrosine and thyroxine by tissue slices *in vitro*, these compounds did not prevent the accumulation of the I^{131} by the slices. McGinty (19) found in rats that, though the organic binding of iodine is completely inhibited by thiouracil feeding, the quantity of iodine in the thyroid gland can be increased by adding potassium iodide to the diet. This iodine, however, was not precipitable with protein and, therefore, presumably still in an inorganic form. It was also noted (20) that the thyroids of rats depleted of iodine by thiouracil treatment would rapidly accumulate iodine if a relatively large dose of ordinary iodide were injected; this freshly accumulated iodine was found to be unattached to protein. Further studies on the iodide ion-concentrating mechanism were greatly facilitated by the finding that it is specifically inhibited by thiocyanate.

It had been noted by Barker (21) that potassium thiocyanate could give rise to goiter and signs of myxedema in man, and it was later found to be goitrogenic in rats (22). However, thiocyanate differed from antithyroid compounds in that its goitrogenic effect could be abolished by increasing the iodide intake. Franklin, Chaikoff, and Lerner (23, 24), using radioiodine, and VanderLaan and Bissell (25), employing ordinary chemical methods, clearly established the effectiveness of thiocyanate in inhibiting the uptake mechanism for iodide. In a detailed study of this

phenomenon VanderLaan and VanderLaan (26) established, as far as current physical and chemical methods would permit, that the iodine accumulated by the thyroid gland, when organic binding is completely inhibited by propylthiouracil, is indeed iodide ion. They showed that thyroid tissue can maintain an iodide ion concentration much higher than that of the circulating blood. This gradient, or, expressed another way, the thyroid/blood iodide ratio, is increased when the thyroid is hyperplastic. Thiocyanate inhibits this concentrating mechanism and within an hour or less after thiocyanate is given, the thyroid/blood iodide ratio has approached 1/1. It is of interest to note that VanderLaan and Vander-

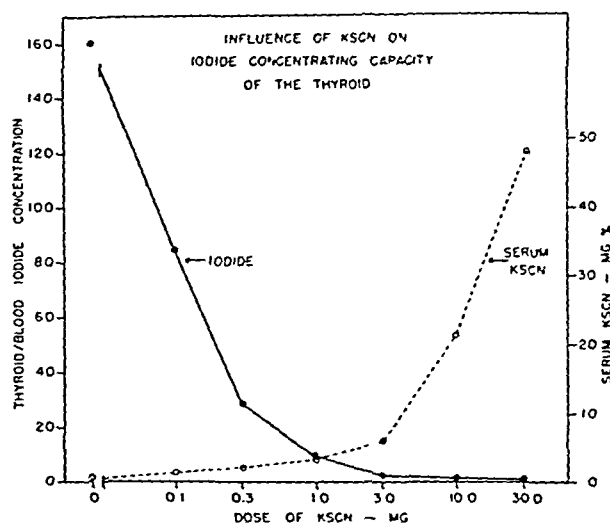


FIG. 1. THE EFFECT IN NORMAL YOUNG RATS OF VARIOUS DOSES OF POTASSIUM THIOCYANATE ON THE THYROID/BLOOD IODIDE RATIO

The animals were injected with 5 mg. of propylthiouracil to prevent organic binding of iodine and were given carrier-free radioiodine two hours, and the potassium thiocyanate one hour before being killed.

Laan found that the use of chemical methods yielded the same results as did radioiodine techniques. The effect on the thyroid/blood ratio of increasing doses of KSCN is shown in Figure 1.

In addition to thiocyanate ion, cyanide and sulfide ions were found by Schachner, Franklin, and Chaikoff (27) to inhibit iodine uptake by thyroid slices *in vitro*. Neither azide, sulfanilamide (27), methylcyanide, or thiourea derivatives (23) inhibited uptake, whereas azide, thioureas, and various aminobenzene derivatives (28)

inhibited the incorporation of the radioiodine into diiodotyrosine and thyroxine.

In the intact animal thiocyanate is the only agent which has thus far been found to inhibit the iodide-concentrating mechanism. Bromide is without effect even when given in large amounts to rat (26) or to man (29). Potassium cyanide, in the small doses tolerated, was ineffective in rats (30).

Quantitative studies in the rat showed that the thyroid/blood iodide ratio averaged 25 in the normal animal and 250 in animals whose thyroids were made hyperplastic by the administration of propylthiouracil (26). Taurog, Chaikoff, and Feller (31) also found values averaging 250 for rats with hyperplastic thyroids and noted that this did not change appreciably over the course of 24 hours following the administration of the tracer dose. Though the calculation may be somewhat in error, it is of interest to note that the values found by Franklin and Chaikoff (18) for iodide uptake in slices of normal sheep thyroid glands bathed in Ringer's solution containing sulfanilamide (cited above) permit one to estimate that the slice/fluid iodide ratio was about 40. By a different method, Wolff and Chaikoff (32) found the thyroid/blood iodide ratio of normal rats to vary between 100 and 290—a value much higher than that given by VanderLaan and VanderLaan. This possibly indicates that the thyroids were somewhat hyperplastic as a consequence of a low iodine intake; though the actual intake was not mentioned in this particular report, other papers from the same laboratory cite the value 0.1 $\mu\text{g.}$ of iodine per gram for the diet usually employed (33). Most laboratory diets contain considerably more iodine than this and animals fed on them would have thyroids in a more nearly "resting" state. Subsequent studies in both man and rat indicate that the iodide gradient is an index of the "activity" of the thyroid. The gradient has been observed to fall rapidly after hypophysectomy and to be increased by thyrotropin (34).

Though thyroid tissue can maintain a high iodide gradient, there is a limit to the total quantity of iodide that it can contain. Appreciable additions can be made to the iodide content of the blood without altering the thyroid/blood ratio, but beyond a critical concentration the ratio progres-

sively falls as the blood concentration is further increased. Taurog, Chaikoff, and Feller (31) found the ratio to be unchanged when 2 $\mu\text{g.}$ or 100 $\mu\text{g.}$ of iodide were given to adult rats. VanderLaan and VanderLaan (26) gave increasing doses of carrier labelled with I^{131} to young rats. No change in the iodide gradient resulted from doses up to 100 $\mu\text{g.}$ of potassium iodide; 300 $\mu\text{g.}$ reduced the ratio to half, and 1000 $\mu\text{g.}$ to less than 1/10 the control ratio. The concentrating mechanism could not be completely saturated, however, and even when 10 mg. were given, the thyroid held more iodide than an equal volume of serum.

Organic binding and hormone synthesis

Iodine compounds in the thyroid. Harington has stated that all the organic iodine in the thyroid can be accounted for in two compounds, diiodotyrosine and thyroxine, basing this conclusion on the results of a series of fractionations of the iodine compounds after alkaline hydrolysis of thyroid gland tissue in progressively stronger alkali (35). These compounds are believed to be present in the gland in peptide linkage as part of a large protein molecule, thyroglobulin, and are neither dialyzable nor directly extractable with butyl alcohol. It has recently been found that a small amount of free thyroxine can be extracted from the rat thyroid gland (36).

About 25 to 30 per cent of the organic iodine in the gland is believed to be in the thyroxine fraction, and in a study of 11 different vertebrates (37), the values were remarkably constant, ranging from 24 to 32 per cent.

If the recent claims of Fink and Fink (38) are correct, however, a radical revision of Harington's thesis would be necessary. Hydrolysis products of thyroid tissue after I^{131} administration were studied by paper partition chromatography. Initially, several radioactive spots were noted which could not be attributed to the known iodine compounds of the thyroid (39), and more recently, both in man and in the rat, the radioactivity in a spot on the paper chromatogram, identified as being due to monoiodotyrosine, was found to be $\frac{1}{3}$ to $\frac{2}{3}$ as intense as that of the diiodotyrosine spot (38).

The iodination mechanism, by which the peptide-linked tyrosyl radicals are iodinated, is

but poorly understood. It is likely that the iodide ion which has entered the gland, and presumably the follicular cell, must be oxidized to I_2 or to IO^- for the iodination to proceed; an oxidative mechanism capable of effecting this step must be sought. A peroxidase enzyme would be a logical possibility, as it would meet the requirements of the high potential involved in the oxidation process. Histochemical evidence of peroxidase activity within the parenchymal cells has been offered by Dempsey (40), challenged by Glock (41), and supported by de Robertis and Grasso (42). It is an attractive hypothesis that H_2O_2 catalyzed by peroxidase converts I^- to I_2 . But the presence of H_2O_2 has yet to be shown, and for that matter, I_2 or IO^- exists in the cell in theory only, or at any rate, has never been chemically identified. The actual persistence of iodine in a tissue cell in either of these forms is highly unlikely, and the transition from iodide through an oxidized form to its incorporation in the benzene ring of tyrosine may occur almost as a single step. In a study of the kinetics of the *in vitro* iodination of tyrosine, Li (43) concluded that, after entrance of the first iodine atom, the second combines immediately. If this process simulates the events in the thyroid gland, the presence of the large amounts of monoiodotyrosine reported by Fink and Fink (38) would be difficult to understand.

Conjugation. A number of facts lend support to the theory proposed by Harington and Barger (44) that diiodotyrosine is the precursor of thyroxine in the thyroid gland. Diiodotyrosine incubated at slightly alkaline pH will be partially converted to thyroxine (45); the iodination of casein gives a high yield of diiodotyrosine and later, after incubation, large amounts of thyroxine can be isolated (46, 47). Observations on the changes with time of the relative specific activities of thyroxine and diiodotyrosine in animals injected with radioactive iodine have yielded results consistent with formulae predicting the relationship of the activities of a substance and its precursor (48).

The formation of thyroxine is thought to occur as the result of the oxidative coupling of two molecules of diiodotyrosine with the elimination of one side chain. The oxidative conditions required for this coupling could potentially be provided by iodine or hypoiodite, or perhaps by what-

ever mechanism produces the oxidation of iodide. It has been pointed out (49) that peroxidase could catalyze this oxidative coupling, but whether a specific enzyme is actually concerned with this step is not known.

The rate at which organic binding and coupling goes on depends on the state of activity of the gland, and is accelerated by thyrotropin (50) and exposure to cold (51), slowed by hypophysectomy (4, 52), and influenced by the *previous* iodide level (36). The rate is also affected by the iodide concentration at any moment (32), as noted below.

An interesting chemical model of the iodination process has been devised by Keston (53). I^{131} in iodide form added to unpasteurized milk failed to yield diiodotyrosine or thyroxine to any considerable extent until xanthine was added to the system, after which 67 per cent of the radioactive iodine could be recovered in these compounds. It is presumed that the xanthine oxidase present in milk liberated H_2O_2 , which either directly, or catalyzed by the peroxidase also present in milk, oxidized the iodide to a more reactive form, which in turn iodinated tyrosyl groups. The enhancing effect of xanthine could be prevented by the addition of thiourea.

Experiments in general demonstrating that the iodination of casein (with elemental iodine) produces diiodotyrosine and thyroxine within the protein make plausible the belief that the process takes place in the thyroid gland at the level of protein molecules. Indeed, diiodotyrosine and thyroxine are formed much more rapidly *in vitro* when protein is iodinated than when the process is carried out with the free amino acid, tyrosine (45, 48).

Extra-thyroidal hormone formation has been suggested by several investigators. If radioactively labelled iodide is injected into thyroidectomized rats, small amounts of the iodine can be recovered in the various tissues of the body as diiodotyrosine and thyroxine (54). The subcutaneous injection of elemental iodine will exert thyroid hormone-like effects (55) and produce considerable iodination of tissue protein (56), but this represents a highly unphysiological situation. The metamorphosis of thyroidectomized amphibians has been found to be stimulated by iodine (57), and the metabolic rates and body weights of thyroidectomized rats have been shown to be slightly

higher on a diet adequate in iodine than on an iodine-deficient diet (58).

The production of cretinism and myxedema by removal of the thyroid, however, would seem to be adequate evidence that if extra-thyroidal production of thyroxine occurs, the quantity falls far below the requirements of the body.

Action of antithyroid compounds. It has been well established by non-radioactive technics that antithyroid compounds of both the thiocarbonamide (thiourea derivatives, etc.) and the aminobenzene (sulfonamides, etc.) types act by inhibiting the formation of thyroid hormone. Numerous studies employing radioactive iodine have confirmed this. Both in tissue slices (18, 23, 28) and in the intact animal (30, 31, 59-62), these substances interfere with the organic binding of iodine, but do not inhibit the thyroid's capacity for concentrating iodide ion. When antithyroid compounds are present in effective concentrations, the incorporation of radioactive iodine into diiodotyrosine and thyroxine is prevented. The first step in hormone synthesis, the iodination of tyrosine, must, therefore, be blocked. In the face of this block, it becomes more difficult to determine whether the second step, the conversion of diiodotyrosine to thyroxine, is also affected, and in fact, it is not really known whether antithyroid compounds influence the latter process. Recently Pitt-Rivers (63) has been able to inhibit the *in vitro* conversion of acetyldiiodotyrosine to acetylthyroxine with a number of antithyroid compounds of both the thiocarbonamide and aminobenzene type (but with amounts of these substances which would probably represent enormous *in vivo* doses).

It has been observed repeatedly that following a single dose of thiouracil (or one of its relatives), or following a prolonged period of administration as well, the resumption of organic binding occurs within a matter of hours after the drug has been stopped (64, 65). If the administration has been continued long enough, a diminution of circulating thyroxine will occur, and an excessive output of thyrotropic hormone by the pituitary gland will have resulted (66, 67). The thyroid gland, under these circumstances, is found to be grossly enlarged, hyperplastic microscopically, and hyperactive as judged by the increased capacity to concentrate radioactive iodide (26). One experi-

ment (68) has suggested a prolonged suppression of the capacity of the rat thyroid to bind radioiodine after a period of thiouracil administration. The turnover rate could, however, have been so fast that the single determination obtained in 48 hours would not necessarily have been conclusive.

A number of possible explanations have been suggested to account for the inhibition of the organic binding of iodine by antithyroid drugs: (1) Thiouracil immediately reduces I_2 *in vitro* at neutral pH, and probably, in this way, inhibits the iodination of casein (69). It could conceivably act in the thyroid either by preventing the oxidation of iodide, or by reducing the oxidation product as rapidly as it is formed. (2) Thiouracil is said to suppress the activity of the peroxidase in the follicular cell (40); and, thus, if this theory of the oxidation mechanism is correct, might act by inhibiting the catalyst of the oxidation, or by serving as a competitive substrate for peroxidase (70). (3) It has been suggested that antithyroid compounds might act by inhibiting the cytochrome-oxidase system (71). The failure of antithyroid compounds to depress thyroid tissue respiration (72), however, makes this possibility remote.

It would seem unlikely, too, that cytochrome-oxidase, or any enzyme which is so widely distributed in the body, should catalyze the oxidation of iodide in the thyroid gland, and not catalyze it in all other tissues as well. We do not, however, know to what factor the thyroid owes its remarkable powers for iodinating proteins. Does a specific oxidative enzyme in the thyroid cell endow it with this property, or does it rather contain only the oxidative potentialities of all cells, but gain its special property through a unique permeability to the iodide ion?

Inhibitory effect of iodide. An observation important to the understanding of the influence of iodine upon the function of the thyroid gland is the striking inhibitory effect of a high concentration of iodide upon the process of organic binding. First demonstrated in 1944 by Morton, Chaikoff, and Rosenfeld (73) for sheep tissue slices bathed with a bicarbonate-Ringer's solution containing varying concentrations of labelled iodide, this phenomenon was later more extensively investigated in intact animals by Wolff and Chaikoff (32, 74). Rats were injected with labelled iodide in doses ranging from 10 to 500 μ g. and the amount

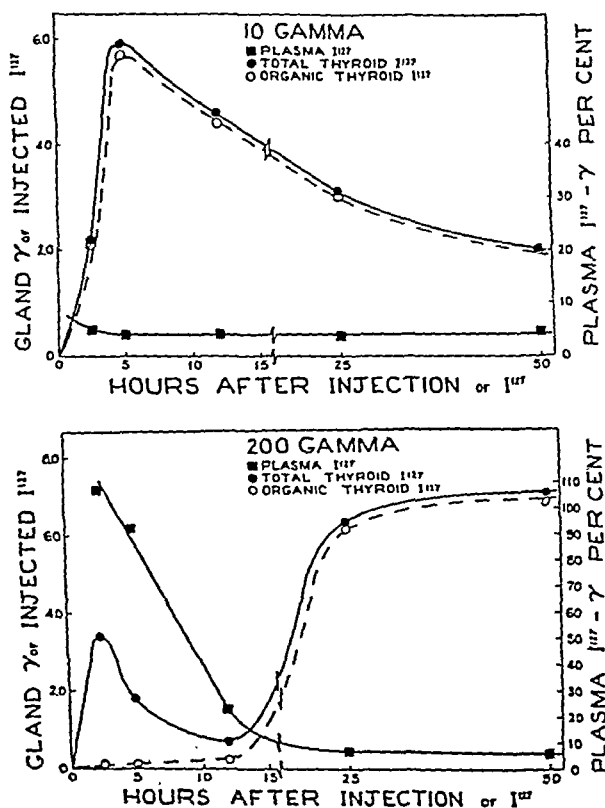
of iodine organically bound calculated from the amount of radioactivity measured in the trichloroacetic acid-precipitable fraction of the thyroid glands. When correlated with the plasma iodide concentration, it was clearly demonstrated that at plasma values above 20 to 35 $\mu\text{g. per cent}$ the organic binding of iodine practically ceased (Figures 2a and 2b). This inhibition could be prolonged for 40 hours from a single, large injection in nephrectomized rats (75).

Recent observations which we have made (76) suggest that, as might have been anticipated, the blocking effect of iodide correlates more directly with the concentration of the ion in the thyroid gland than with the plasma concentration. Evidence for this was obtained from the following findings: (1) The rats used in the experiments of Wolff and Chaikoff (32) maintained an exceed-

ingly high thyroid-serum iodide ratio, probably due to a low dietary iodine intake. Using rats which maintained a lower ratio, we found that considerably greater amounts of iodide were required to cause inhibition of organic binding. (2) If rats injected with a blocking dose of potassium iodide were also given a dose of potassium thiocyanate, which, as indicated earlier, would lower the thyroid-serum ratio, it was found that organic binding could then occur. Under these circumstances, the thyroid iodide concentration had been lowered without changing appreciably the high plasma values.

One is tempted to apply this newer information concerning iodine influence on thyroid function to explain the well-known salutary effect of iodine in Graves' disease. In this condition, when untreated, the gland is enlarged and markedly hyperemic; the parenchymal cells are columnar and tall; the follicles are irregular with many papillary projections into the lumina, which are considerably smaller than normal. The colloid appears decreased in amount, is more liquid than the usual consistency, and the organic iodine concentration of the gland is markedly diminished. Under treatment with iodine (in amounts which are distinctly pharmacological rather than physiological), improvement in clinical signs and symptoms frequently ensues, and local changes occur in the thyroid that might reasonably be described as "involutional." The hyperemia diminishes, the follicular cells become flattened, the follicle is rounded, and its colloid content appears increased. In addition, the organic iodine concentration is greatly increased.

Wolff and Chaikoff (32) have regarded the blocking effect of iodide as a "homeostatic regulator" by means of which "the formation of toxic amounts of thyroid hormone is prevented" and believe further that this mechanism explains in part, at least, the beneficial effect of iodine in Graves' disease. To involve this mechanism alone, would, of course, leave unexplained the fact that the organic iodine content of the thyroid, both in the normal animal and in Graves' disease, increases with iodine therapy. It is interesting to note that the data presented by Chaikoff and co-workers (32, 73) demonstrate, though it is not pointed out by the authors, that below the iodide concentration values necessary for inhibition, the absolute



FIGS. 2a and 2b. IODIDE INHIBITION OF ORGANIC BINDING

Groups of rats injected with 10 $\mu\text{g.}$ (Figure 2a) and 200 $\mu\text{g.}$ (Figure 2b) of iodide, respectively, as potassium iodide labelled with I^{131} . Inhibition of organic binding (dotted line) persisted until the plasma iodide level fell to about 20.0 $\mu\text{g. per cent}$ (from Wolff and Chaikoff [32]).

amounts of iodine organically bound increase with increasing amounts of iodide present. This phenomenon has also been observed by us in the rat (76) and by Stanley (77) in man. This point would also be consistent with older observations (33, 78) that the organic iodine content of animal thyroids increases with increasing dietary iodine intake, and that intermittent iodine therapy may increase thyrotoxicity (79).

The rapid inhibition of organic binding by a large concentration of iodide is perhaps the best single piece of evidence of a direct effect of iodine upon the function of the thyroid gland. Though it may be argued that an action through the inhibition of thyrotropin has not been ruled out, the site of inhibition would, in any case, have been defined. The influence of iodine upon the size, and both gross and microscopic appearance of the thyroid treated with thiouracil (19, 80), and upon the gland in hyperthyroidism, further suggests a direct effect.

In the reverse situation, in which stimulation of the thyroid occurs as a result of iodine deficiency, it is difficult to know whether the lack of iodine *per se* acts as a stimulus to the gland, or whether this phenomenon is entirely secondary to increased pituitary stimulation.

Studies using the radioautographic technic were first made on thyroid tissue by Hamilton, Soley, and Eichorn (81) in 1940, in experiments which revealed increased radioactivity in hyperplasia and an absence of activity in the thyroid cancer tissue studied. Subsequent studies have confirmed the idea that information about the formation and storage of organic iodine compounds could be obtained by this method, and refinements in technic (82-87) have made very detailed observations possible.

By use of radioautographs, the accumulation of radioactive iodine has been shown in the stolon septum in the ascidians, *Styela* and *Ciona*, as well as in *Amphioxus* (88), in the endostyle of the lamprey larva (89), in the thyroid of the parrot fish (90), and in the thyroids of 10 mm. tadpoles (91) and 18-day-old rat embryos (92).

Leblond and Gross have studied the effects of low-iodine diet and hypophysectomy in rats in this manner (36). Whereas the autographs of rats previously maintained on 2.0 μg . of iodine per day showed the major concentration of radioactiv-

ity in the colloid of thyroid glands removed as early as two minutes after the injection of I^{131} , those of rats which had been maintained on a 20.0 μg . per day iodine intake, showed the radioactivity chiefly within the follicular cells one hour after injection, but almost entirely in the colloid 24 hours after injection. In hypophysectomized animals, however, despite a low iodine intake, the radioactivity was largely in the follicular cells as late as 24 hours after the I^{131} was given. Thus, these three groups showed three distinct rates at which the processes of binding and storage were proceeding. It would seem likely from the photographs reproduced that the iodination of protein took place in the follicular cells, after which it was extruded into the lumina of the acini.

It should be remembered in interpreting these autographs that iodide ion and any dialyzable iodine compounds, would be washed out in the fixing and processing of the tissue sections as generally practiced (93), and only radioactive iodine compounds in proteins, or attached to proteins, would be influencing the autograph obtained. In this regard, the failure to obtain autographs from thiouracil-treated rats (94) could be a case in point; this occurred despite a considerable uptake of inorganic radioactive iodine by the glands.

Leblond and Gross (36) also point out that their autographs indicate that all thyroid cells are active at all times and that all the cells in any one follicle are functioning to the same degree, although there may be different degrees of activities in different follicles. In general, the center of the rat thyroid gland appears to be more active than the periphery, and the authors have also noted a correlation between activity estimations by the radioautographic technic and those based on staining reactions.

It has always been somewhat difficult to picture the mechanics by which thyroglobulin is secreted into the colloid by the thyroid cell, and then later resorbed and secreted into the blood stream. By an interesting analogy, Leblond and Gross help to make this process seem more reasonable: "It may be helpful for the understanding of the follicle function to remember its entodermal origin and to venture a comparison with the intestinal epithelium, which secretes protein (enzymes) into the lumen in a direction consistent with the histological polarity of its cells, and at the same

time resorbs from the lumen smaller molecules resulting from digestion (amino acids —). Similarly, the thyroid epithelium secretes a protein (thyroglobulin) into the follicle lumen and at the same time resorbs smaller molecules, probably thyroxine itself" (36).

The function of human thyroid nodules has also been studied by radioautographs (95-97), at times with concomitant chemical and histological studies, as well as quantitative radioactivity measurements (98,99). In the majority of the reported cases, the nodules have been considerably less active than the surrounding thyroid tissue, even in patients with clinical thyrotoxicosis.

Hormone secretion

That the essential molecule of thyroid hormone is thyroxine has been well established since the pioneering work of Kendall (100) and of Harington (44). Injected in pure crystalline form, it produces metabolic effects which no other known substance can, in comparable amounts, effect. But granting that thyroxine is an essential constituent of the thyroid hormone, some workers have believed that the hormone was a peptide or polypeptide containing this essential compound. Harington, by 1944, (101) was satisfied that the peptide concept was no longer necessary. The concept of thyroxine as the hormone *per se* has received further support from the work of Taurog and Chaikoff (102), who showed that almost all the organic iodine in plasma behaves like thyroxine in that it: (1) precipitates with the protein, (2) from which it cannot be dialyzed, (3) but from which it is readily extracted with butyl alcohol, (4) in which it remains after extraction with 4N NaOH — 5 per cent Na_2CO_3 . In addition, when a rat has labelled its plasma organic iodine from a previous injection of I^{131} , the radioactivity follows added thyroxine carrier. The carrier thyroxine may be recrystallized to constant specific activity, and the radioactive portion resembles thyroxine in its distribution between two immiscible solvents.

Separations of plasma protein fractions have indicated that the largest portion of the protein-bound iodine is carried by albumin, while the highest concentration is in the alpha-globulin fraction, and the lowest in the gamma globulin (102, 103).

If we accept thyroxine as the thyroid hormone, however, a paradox results from the repeated ob-

servations that the effectiveness of fed thyroid extract is greater than can be accounted for by its thyroxine content. This conclusion has been reached by experimenters using a variety of criteria for evaluating the hormonal activity, including the effect on the metabolic rate (104), the suppression of pituitary thyrotropin secretion (105), and the influence on metamorphosis of amphibia (106).

"Proteolytic enzyme." How does thyroxine, which is present in the colloid of the thyroid gland as part of the thyroglobulin molecule, reach the blood as free thyroxine? De Robertis (107) has removed colloid from the follicle lumen by microdissection and tested its proteolytic activity on the protein edestin. Proteolysis occurred with an optimal yield at about pH 4, suggesting a cathepsin as the active enzyme in the experiment. The ubiquity and non-specificity of this type of enzyme, however, makes it a somewhat unsatisfying answer to the problem. It is, nonetheless, of considerable interest that pretreatment of the animal with thyrotropin increased the proteolytic activity and that prolonged iodine treatment, and hypophysectomy (108), decreased it. In addition, the enzyme content was found to be elevated in glands from thyrotoxic patients. One wishes that in this work the results of proteolysis without added protein had been presented, since surely there must have been present a sufficient quantity of protein in the form of the natural substrate in the colloid itself.

Protein-bound iodine of the plasma. Taurog and Chaikoff (102), as a result of their finding that the protein-bound iodine is chiefly in the form of thyroxine, have concluded that the thyroid preferentially secretes thyroxine, and that "only a small fraction of the gland's iodine leaves the gland as diiodotyrosine," although perhaps two-thirds of the organic iodine of the thyroid is in the latter form. This assumption is perhaps unnecessary, however, since it has been found repeatedly that diiodotyrosine is rapidly destroyed in the body. Thus, Leblond and Süe (4) discovered that 30 minutes after the injection of 10 mg. of radioactive diiodotyrosine into rats, 97.5 per cent of the radioactivity in the blood was in the form of iodide ion. No comparable rate of destruction of thyroxine has been demonstrated.

In this regard, it is interesting to note, too, that soon after a dose of radioactive iodide is given,

when the radioactivity first appears in the protein-bound iodine, it is predominantly in the diiodotyrosine-like fraction; later, it is chiefly in the thyroxine-like component (50).

Factors influencing the rate of hormone formation and discharge

The rate, for a given plasma iodide level, at which thyroid hormone is formed and discharged is a function of the general level of activity of the gland, as is the iodide-concentrating power. That these processes may go on independently of each other is neatly demonstrated when, in the presence of an antithyroid drug, the power to concentrate iodide and discharge hormone is retained while hormone formation is prevented, while with thiocyanate, hormone formation and discharge can occur, although the iodide-concentrating power has been lost. Hypophysectomy greatly depresses all three processes, whereas thyrotropin raises the general level of activity of the gland. Thus, beginning eight hours after the injection of thyrotropin in man, a striking increase in the thyroid/serum iodide ratio occurs, as demonstrated by simultane-

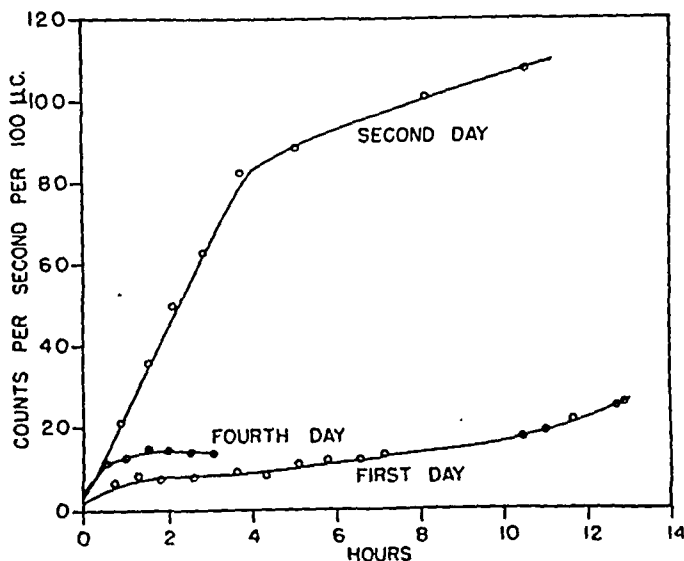


FIG. 3. THREE SUCCESSIVE TRACER STUDIES WITH RADIOIODINE IN A NORMAL HUMAN SUBJECT WHO HAD TAKEN 5 GRAINS OF THYROID DAILY FOR SEVERAL MONTHS

A single injection of 15 mg. of thyrotropin was given $4\frac{1}{2}$ hours before the first dose of radioiodine; its effect was noticeable toward the end of the first day and was maximal during the first four hours of the second day; the thyroid had returned to its suppressed state by the fourth day (redrawn from Stanley and Astwood [109]).

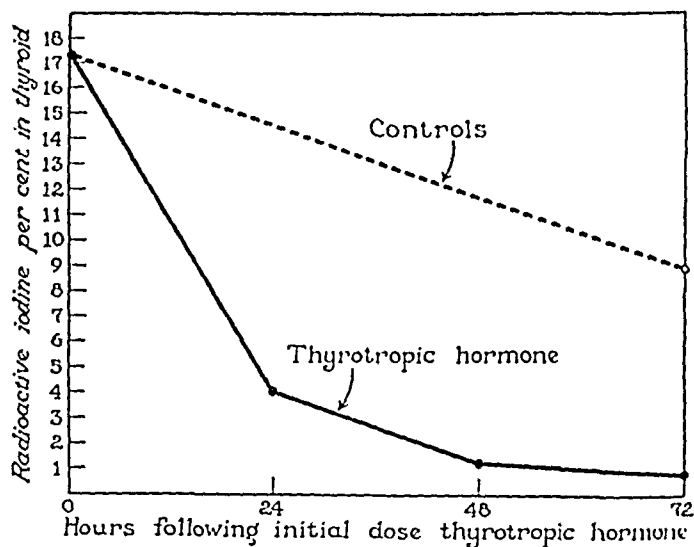


FIG. 4. INFLUENCE OF THYROTROPIC HORMONE ON THE RATE OF DISCHARGE OF ORGANICALLY BOUND RADIOIODINE BY THE CHICK THYROID

(From Keating, Rawson, Peacock, and Evans [110]).

ous measurements of radioactivity in the anti-thyroid-blocked gland and the serum (109). The rate at which radioactive iodine is incorporated into thyroid organic iodine is markedly hastened by thyrotropin (4, 50) (Figure 3), as is the rate at which radioactivity appears in the protein-bound iodine (50). It is well known that injections of thyrotropin rapidly deplete the organic iodine content of the thyroid of animals, indicating that, under the conditions of the experiments, at least, the rate of iodine binding does not initially keep up with the rapid rate of discharge. Using radioiodine, Keating, Rawson, Peacock, and Evans (110) found that thyrotropin accelerated the loss of protein-bound iodine from the thyroid gland (Figure 4). Other changes after thyrotropin, such as the liquefaction of the colloid and the reduction in colloid in the follicles, are consistent with the concept of increased proteolytic activity.

Many other factors undoubtedly influence the level of activity of the gland, including the dietary iodine intake and environmental temperature. Leblond *et al.* (51) found that rats exposed to an environment at 0°C . have a higher radioiodine uptake than rats at 22° to 26°C . (control T°), and by the goiter-prevention method (111), it can be shown that a greater amount of administered thyroxine is required in a cold, than in a warm environment.

The fate of thyroxine in the body

When radioactive thyroxine, labelled in the 3',5' positions, is injected into rabbits, very little is found in the thyroid, or in the foetus of a pregnant animal, but a considerable amount rapidly appears in the urine, and the iodine content in the bile is 50 times as great as after an equal amount of iodine as iodide ion (112). *dl*-Thyroxine, labelled as in the preceding experiment, has been injected into rats and its distribution at two and 24 hours studied in more than 40 organs and tissues (113). When the dose was 0.8 mg., approximately 25 per cent of the administered thyroxine was destroyed in two hours and 50 per cent in 24 hours, by which time 80 to 95 per cent of the iodine content had been eliminated from the body. The major route of elimination was via the liver and bile, and within 24 hours, 80 per cent of the injected dose was recovered in the feces, to a large extent as thyroxine. A part of this thyroxine came from direct excretion by the gastrointestinal mucosa. About 11 per cent of the iodine was recovered in the urine after this interval as inorganic iodine, while the organic iodine content of the urine was very small. Within two hours of the injection, 50 per cent was found in the gastrointestinal tract (including liver and pancreas), and not more than 2 per cent could be recovered in the entire circulating plasma volume.

These results on the fate of thyroxine in the body apply to large, unphysiological amounts, and much less is known about the normal course of events. After thyroidectomy in rats, it takes three days for the protein-bound iodine to fall to one-third of its initial level (114), suggesting a much slower rate of loss of thyroxine at physiological levels. However, in an experiment in which rat plasma containing labelled protein-bound iodine was injected intravenously into dogs, it was calculated that the protein-bound iodine was completely replaced every four to seven and one-half hours (115).

Radioiodine in clinical investigation and diagnosis

Investigations on animals have been facilitated by the use of radioiodine and many studies which could be done with ordinary iodine were rendered

much less laborious with tracer technics. In clinical studies, however, tracer methods have made it possible to carry out investigations on thyroid function of a kind which was not possible by other methods. Several different technics have been evolved to estimate the rate of iodine utilization by the thyroid gland, and these have aided in the diagnosis of myxedema and hyperthyroidism, and have furthered the study of simple goiter. These methods have also been applied to the study of factors which influence thyroid function in man such as antithyroid compounds, thiocyanate ion, iodide ion, thyroid administration, thyrotropin, and the ingestion of certain foods.

The urinary excretion of a tracer dose has been used as an indirect measure of thyroid activity by making use of the fact that a proportion of the tracer dose which does not appear in the urine is retained in the thyroid gland. Hamilton and Soley (12), and later Hertz, Roberts, and Salter (13), showed that the urinary excretion of radioiodine had diagnostic value. Rawson *et al.* (116) have shown, by this method, that thiouracil treatment permitted the excretion of the major portion of a tracer dose in the urine, while thyrotoxic patients and one patient with a large hyperplastic non-toxic goiter excreted less than one quarter of the administered radioiodine during 48 hours. Measurements on urinary excretion have also proved useful in determining the amount retained when large doses of radioiodine are used in the investigation and treatment of thyroid tumors (117-119) and in the treatment of hyperthyroidism with radioiodine (120, 121).

Despite the difficulties involved, several observers have found the urinary excretion method of considerable value in the diagnosis of hyper- and hypothyroidism. Skanse (122) measured the amount of radioiodine excreted in the urine during the 48 hours after the administration of 100 μ g. of iodide labelled with I^{131} and found that hyperthyroid patients uniformly excreted less than normal individuals. Oshry and Schmidt (123) measured the total quantity of radioiodine excreted during the first 24 hours after a tracer dose. The values observed are shown in Table I.

A detailed study of the rate of urinary excretion of radioiodine by Keating, Power, Berkson, and Haines (124) has permitted an accurate in-

TABLE I

	Skanse (122)		Oshry and Schmidt (123)	
	No. of cases	Range of radioiodine excretion 48 hours	No. of cases	Range of radioiodine excretion 24 hours
Normal	15	52.7 to 84.1	23	42 to 80
Myxedema	6	72.4 to 91.7	10	48 to 93
Hyperthyroid	25	6.2 to 32.3	34	5 to 43

terpretation of the findings obtained by this method; they have shown that, as the blood is cleared of radioiodine by its excretion in the urine and its incorporation into the thyroid gland, the quantity excreted in the urine progressively declines. Consequently, from the quantities excreted in the urine, measured at frequent intervals, the rate of disappearance of radioiodine from the blood can be calculated.

Keating *et al.* made use of the expression,

$$Q = Q_t(1 - e^{-rt}),$$

where Q is the fraction of the tracer dose excreted in the urine in time t ; Q_t the total quantity to be excreted; and r , a constant defining the rate of disappearance of radioiodine from the blood. The disappearance rate, r , was computed, then, from the formula,

$$r = \frac{\log(Q_t - Q)}{t \log e},$$

or as the slope of the straight line plot of $\log(Q_t - Q)$ against t . As Q_t is the fraction to appear in the urine, $1 - Q_t$ would be the fraction retained in the body. Therefore, the urinary excretion rate becomes:

$$Q_t \times r$$

and the rate of disappearance elsewhere, the "collection rate" is:

$$(1 - Q_t) \times r.$$

This last expression is an index of the rate of incorporation of the tracer dose into the thyroid gland (Figure 5).

The validity of these concepts was established by similar calculations made by estimating the blood disappearance rate on the basis of actual measurements on blood samples, and by calculations using measurements of the accumulation of radioiodine by the thyroid gland. It was even possible to use the decreasing radioactivity of the thigh as a measure of the disappearance rate.

In individuals with thyrotoxicosis the disappearance rate may be extremely rapid and these calculations then do not so nicely follow mathematical prediction. Presumably, at least two additional factors complicate the picture here: (1) The thyroid collection rate is not uniform because it is the resultant of two rates, the rate of establishment of iodide ion equilibrium and the rate

of organic binding, and (2) the significant rate of excretion of iodine-containing compounds by the thyroid gland.

A certain proportion of the radioiodine which fails to appear in the urine cannot be accounted for by the amount which is measured in the thyroid gland. In earlier experiments this "unaccounted for" fraction was very large (13), due probably to technical difficulties of measurement. Even with refined technics, however, some 10 to 20 per cent of the dose is not accounted for (125). It would be expected that a certain small fraction of the administered dose would be contained in the intestinal tract, and some of this might be lost in the stools. It is possible that a small fraction would be excreted by the sweat glands. The observations of Keating *et al.* (124) cited above show that when the turnover of iodine by the thyroid is very rapid, a slow excretion of radioiodine continues for some days after the tracer dose is given. Consequently, another fraction of the "unaccounted for" radioiodine could be that which has been secreted by the thyroid in the form of thyroxine and temporarily stored in body tissue. Direct measurements on the quantity of radioiodine in the thyroid gland are probably more accurate than estimates based on urinary excretion and are far simpler to carry out.

Direct measurements of the collection of iodine by the thyroid gland, using appropriate apparatus, is a more convenient and more versatile method for thyroid studies in man. Methods and detecting devices are constantly being improved, but as yet strictly precise, absolute measurements are not possible. However, for most purposes any one of several currently used technics is satisfactory (125-128), and the method of choice depends upon the nature of the study. When it is desired to determine only the total quantity of radioiodine accumulated, a single measurement at 24 to 48 hours after the tracer dose is sufficient; when more detailed information is desired, measurements can be made at frequent intervals. During the early minutes or hours after a tracer dose is given, the radioiodine is distributed throughout the body and provides a diffuse radiation which complicates the measurements on the thyroid gland. The greater the distance between the detector and the gland, the greater is the difficulty of shielding off the radiation from the rest of the body. When this error

is minimized by placing the detector as close to the thyroid as possible, errors due to positioning and to size, location, and shape of the thyroid gland become maximal. These considerations imply that when absolute values are desired, measurements must be made at a distance and preferably at a time when the body in general contains little radioiodine. When the observation of rapid changes, occurring soon after the tracer is given, is more important than information on the number of microcuries in the gland, it may be necessary to sacrifice the added accuracy of distance in favor of the simpler shielding problem of closer placement.

Curves depicting the collection of radioiodine by the thyroid gland resemble those obtained by Keating *et al.* (124) for cumulative urinary excretion. The course of uptake is apparently complex, and no simple mathematical expression is

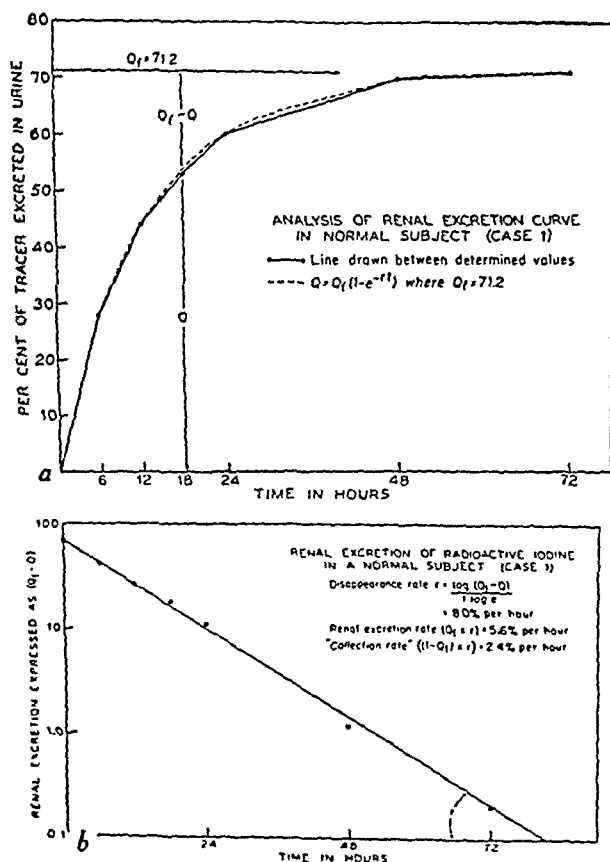


FIG. 5. DETERMINATION OF RATE OF DISAPPEARANCE OF RADIOIODINE FROM THE BLOOD (r) BY ANALYSIS OF THE RENAL EXCRETION CURVE

(From Keating, Power, Berkson, and Haines [124]).

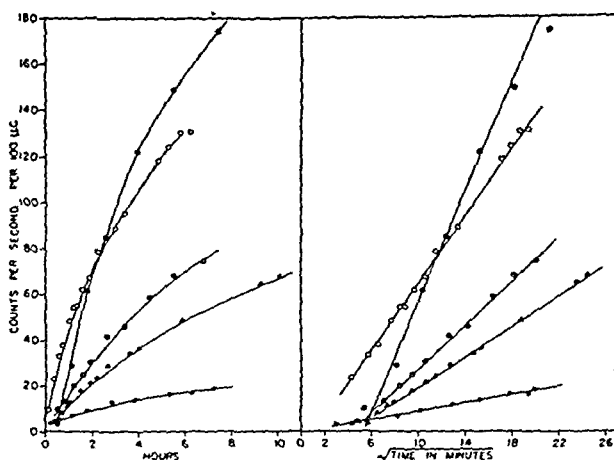


FIG. 6. THE COURSE OF RADIOIODINE ACCUMULATION IN THE THYROID GLANDS OF THREE NORMAL AND TWO THYROTOXIC INDIVIDUALS

The figure on the right illustrates the approximately linear relationship when the abscissa is a square root scale.

applicable to all individuals. The formulation of Keating *et al.* provides an approximate description of the pattern seen in most normal persons. In other normal persons and in many abnormal individuals the relationship fails to hold. Another approximation (65) which describes the curve of collection during the first part of its length is the simple relationship

$$Q = G\sqrt{T},$$

where Q is the quantity of radioiodine in the thyroid gland at time T ; and G is a rate constant which serves as an index of the rate of uptake. This expression describes the course of uptake quite accurately in normal persons for the first eight or ten hours after the tracer is given. In hyperthyroidism and in certain instances of goiter, when the collection is rapid, deviation from the parabolic curve occurs sooner, and in some instances the curve of uptake bears little resemblance to a parabola. On the other hand, when the rate of collection is slow, the curve of uptake follows the parabolic curve quite closely for as long as 24 hours (Figure 6).

This method of expressing the rate of accumulation suffers the disadvantages of: (1) Incomplete description of the whole process, (2) marked and early deviations in certain cases, and, (3) obscure mathematical significance. It has the advantage, however, of providing a measurement

of rate within an hour or two of the beginning of the tracer study, and it is the only satisfactory method thus far devised which makes it possible to define the accumulation rate early enough to allow studies on extraneous factors which induce rapid changes in the rate of accumulation.

The rate as determined in this way is not proportional to the total accumulation and is less suitable than the latter as a diagnostic method for hyperthyroidism. Either method is suitable when the diagnosis of myxedema is in question. Neither of these methods is entirely reliable for diagnosis, however, as the range of variation in normal individuals overlaps at either end with myxedema and thyrotoxicosis. Erroneously low accumulation rates and low total uptakes follow iodine or thyroid medication, the administration of compounds used for cholecystography, and the installation of iodized oils into the lungs, subarachnoid space, or other body cavities. Rapid rates and large total uptakes within the hyperthyroid range are sometimes seen in apparently normal persons and in some instances of non-toxic goiter. Despite these limitations, the two methods are valuable and frequently provide strong evidence for or against a diagnosis of myxedema or hyperthyroidism.

Werner, Quimby, and Schmidt (129) have extensively investigated the total collection of iodine by the thyroid by direct measurements 24 hours after the tracer dose. The data are shown in Table II from which it may be seen that little

overlap between the normal values and those of hyperthyroids was encountered.

Iodide ion. Radioiodine, used in conjunction with antithyroid compounds, has made it possible to study the iodide ion concentration by the thyroid gland in human beings and the effects thereon of thiocyanate (29). In man, thyroid hyperplasia, as seen in hyperthyroidism and in certain non-toxic goiters of recent development, is associated with a greatly increased capacity of the gland to hold iodide ion and quantitative measurements of this phenomenon have diagnostic value. Potassium thiocyanate given orally in doses of about 1 gram greatly interferes with the ion-concentrating mechanism and virtually all I^{131} present in the gland in ionic form is lost within an hour.

The organic binding of iodine by the thyroid can be completely or very nearly completely inhibited by the administration of effective doses of an antithyroid compound. Mercaptoimidazole has been used for this purpose because of its high activity in man and its prolonged action, and more recently 1-methyl-2-mercaptoimidazole has proved to be convenient because of the small size of the doses that need be given (130). When radioiodine is administered after the thyroid is fully under the influence of such compounds, it serves to label the iodide ion of the body fluids and the thyroid gland. As far as one can tell, this iodide ion does not enter into any chemical reaction in the body and interpretations are, there-

TABLE II *

Radioiodine accumulation in 24 hours		Normal subjects	Hyperthyroidism	Nontoxic goiter	Malignant exophthalmos	Eosinophile adenoma	Chromophobe adenoma	Addison's disease	Cushing's syndrome	Obesity	Anorexia nervosa
H I G H	<i>per cent</i>										
	50-	0	57	0	3	0	0	0	0	0	
	40-49	2	29	2	1	2	0	0	0	0	
	35-39	4	5	2	0	1	0	0	0	0	1
N O R M A L											
	30-34	8	2	2	0	1	0	1	0	3	1
	20-29	24	2	3	3	2	1	1	0	3	1
	10-19	18	2	1	1	4	5	3	1	1	3
L O W											
	0-9	1	0	1	0	0	3	0	1	1	0

* Compiled from data of Werner, Quimby, and Schmidt (129).

fore, greatly simplified. Within an hour or two after the tracer dose is given, the radioiodine seems to have distributed itself throughout the body's iodide and this state of equilibrium is maintained for as long as detectable quantities of radioiodine remain in the body. The ion is lost from the body almost exclusively through the urine, and, as a certain fraction of what remains is lost during each succeeding interval of time, the rate of loss is an exponential function. Thus, if one plots the log of the blood concentration against elapsed time, a straight line results. Now, as mentioned above, the thyroid gland can steadily maintain a concentration gradient of iodide ion, and equilibrium conditions here are also quickly established. It follows, therefore, that the maximal thyroid content of radioiodide is to be observed within an hour or two of the tracer dose; thereafter the radioiodide in the thyroid gland declines slowly in a regular logarithmic fashion and becomes undetectable in two to four days. Calculations can be made in man of the thyroid/blood iodide ratio, and, while these are subject to considerable error from patient to patient, the calculated ratio remains remarkably constant throughout the course of a

tracer study on any individual. The decline in the thyroid radioiodide parallels closely the decline in blood radioiodine concentration, so that the ratio remains the same for as long as it can be measured, provided, of course, that sufficient antithyroid compound be given to prevent any change in the chemical state of the iodine.

In the diagnosis of hyperthyroidism, tracer studies of the thyroid iodide content have proved to be more reliable than measurements of the total iodine uptake. There is a greater difference between the hyperthyroid gland and the normal gland, and, with the exception of certain hyperplastic non-toxic goiters, there appears to be no overlap of the ranges of normal and hyperthyroid persons. Furthermore, as might have been predicted from the animal experiments cited above, extraneous ordinary iodide has much less influence on the iodide-concentrating mechanism, and doses of potassium iodide up to 10 mg. or more can be given without altering the thyroid radioiodide. The large collection of iodide ion exhibited by the thyroid in hyperthyroidism and by certain non-toxic goiters can be reproduced in normal individuals by the injection of a single, large dose of thyrotropin (109).

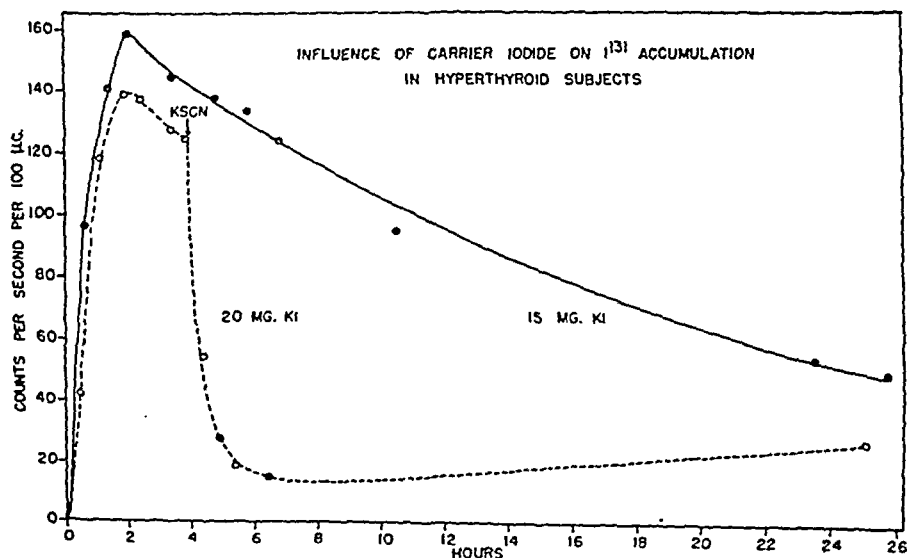


FIG. 7

In the presence of a large carrier dose, the curve of radioiodine uptake and discharge represents largely iodide ion. Thus, the radioactivity in the thyroid may be discharged with thiocyanate (dotted line graph). The carrier doses in the above cases are in the range which inhibits organic binding, but are well below the amount required to depress the thyroid/serum iodide ratio.

If further study confirms the finding that a sudden increase in blood iodide inhibits the organic binding of iodine, another and perhaps better method will be provided for measuring the thyroid iodide for diagnostic purposes. In this instance an appropriate dose of carrier iodine would replace the antithyroid compound. For some hours after the administration of 10 mg. or more of labelled potassium iodide, the quantity of radioiodine fixed in the thyroid is very small. However, within an hour or two of such a dose there is a maximal accumulation of iodine which presumably is free iodide ion, as it is readily discharged by a dose of potassium thiocyanate (Figure 7). The quantity of iodide accumulated under these conditions is independent of the dose of carrier iodide within wide limits. One could, therefore, use a constant dose of carrier with the diagnostic radioiodine in a quantity large enough to reduce the proportion that will be organically bound to a very small value and yet not so large as to impose a limit to the concentrating mechanism. Measurements need only to be made until the early maximum is reached, a period of one to three hours. This technic would be essentially that which Hamilton and Soley used in their first experiments in 1940 (126). The method would have the advantage of simplicity and the time required to carry out the test would be reduced to a minimum. One might anticipate that, though a small fraction of the iodide would be bound, this would be so small in comparison to the quantity concentrated as iodide that it would not seriously interfere with the test.

Antithyroid compounds can readily be studied in normal human beings by making use of the $Q = G\sqrt{T}$ relationship (65). Frequent determinations over one to two hours following the oral administration of a tracer dose permits one to calculate the value of G and to plot the course of uptake with some assurance. A test dose of compound is then given and a significant deviation from the predicted course of events can with reasonable certainty be ascribed to the antithyroid compound. This method has given useful information on the relative antithyroid activities of several compounds and has led to the development of agents which are many times more effective in man than is thiouracil. Some information can be

gained, in this way, on the duration of action as well as on the minimal effective dose.

Foods of possible or potential influence on thyroid function have also been tested in this way (131). The technic used was the same as that described above, except that a full meal of a single food was given instead of a dose of antithyroid compound. A number of foods, especially vegetables, were found to have slight effects on the rate of radioiodine collection by the thyroid gland, and some, such as turnips, were strongly inhibitory. These studies led to the isolation and characterization of an active antithyroid compound in turnip, a substance which was found to be widely distributed among the plants of the brassica genus (132).

Thyroid administration has been observed to have no significant effect upon the accumulation of radioiodine by the thyroid gland during the first ten hours following a single dose. A large dose was found to be definitely inhibitory by 24 hours (133), however, and prolonged treatment in normal individuals virtually abolished the uptake of radioiodine (109, 129).

Thyrotropin was likewise without detectable effect until some eight to 14 hours had elapsed after a single injection. Then a marked increase in the rate of uptake was noted and this lasted for several days (Figure 3). The negligible uptake resulting from prolonged administration of thyroid could be restored to normal by an injection of thyrotropin (109).

Means (134), writing in 1948, has stated, in reference to the use of radioactive iodine in the field of research, that he had "at the time of its introduction, predicted that its usefulness in this field would be greater than in that of therapeutics. Now, after an elapse of ten years, it can be said that the usefulness of radioactive iodine in thyroid research has been firmly established."

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RADIOIODINE THERAPY IN GRAVES' DISEASE

A REVIEW

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HISTORICAL

The discovery of radioactive isotopes of iodine by Fermi (1) in 1934 was followed in 1938 by the use of these tagged atoms in studies of the physiology of the thyroid in human and other animals (2, 3). The selective deposition of radioiodine in the thyroid and the known effectiveness of radiation therapy in selected patients with Graves' disease soon led to the use of two radioactive isotopes of iodine in the treatment of thyrotoxicosis: I^{130} with a half-life of 12.6 hours and I^{131} with a half-life of eight days. Hertz and Roberts (4) first used this method of therapy in the spring of 1941. Hamilton and Lawrence (5) treated their first patients in the fall of 1941 and Chapman and Evans (6) took over the work of Hertz and Roberts (7) in 1943. The earliest reports to allow an even tentative appraisal of radioiodine therapy were published by the latter groups of authors in 1946 (6, 7). In 1948, Soley and Miller (8, 9), published their experiences and later in the same year two other groups recorded their results (Haines, Keating, Power, Williams and Kelsey [10]; Werner, Quimby and Schmidt [11, 12, 13]). Prinzmetal and his coworkers (14, 15) and Crile (16) contributed their material in 1949.

PERSONNEL

Radioiodine as a therapeutic agent in Graves' disease is still in the experimental stage and much remains to be learned about its clinical use, its immediate biological effects, and its long-time effects, both upon the thyroid and upon the patient as a whole. Certain skilled personnel are essential to any program dealing with radioiodine since it is a form of radiation therapy. Such a program may be divided into three general phases: radiological, physical and clinical. There can be no doubt but that the very important problems of radiation

dosimetry and biological effects of ionizing radiations should be handled by a radiologist. The technical problems relating to the physical measurements of radiation, to the maintenance and operation of electronic equipment, and monitoring of the laboratory areas are most capably handled by a physicist. As is always true in medicine, it is important to render care to the patient as a whole. In prescribing a relatively new and powerful remedy, the clinician must make frequent and careful appraisals during the course of treatment and in the long term follow-up of treated patients.

RADIATION EFFECTS OF I^{131}

Although no serious toxic effects have been observed as yet in patients receiving I^{131} therapeutically, thyroidal and extrathyroidal changes have been found in experimental animals.

Gorbman (17) gave young mice 3-55 mc. per kg. of I^{131} to study the effects on the thyroid and adjacent tissue. Within the first day the striking effect was periglandular edema with infiltration by lymphocytes, polymorphonuclear and mast cells. Changes in cells of tracheal epithelium were common. At the end of two days with large doses (53 mc. per kg.) the thyroid was an eosinophilic mass with recognizable follicles only in the peripheral parts of the gland. After three days destruction of the thyroid was complete when 50 mc. per kg. were administered and minimal with the lowest dosage. Comparable changes were noted in parathyroids. Over 20 mc. per kg. caused histologic damage to the recurrent laryngeal nerves. At the end of 24 days as little as 3-5 mc. per kg. caused extensive destruction of the thyroid; 18-22 mc. produced complete thyroid destruction. After an interval of 120 days, the thyroid was only a shrunken fibrous band, except in those animals who had received the smallest amounts of I^{131} .

The University of California investigators (8, 9) found extensive but not complete thyroid destruction by the 30th to 40th days in rabbits from the effects of 300 μ c. per kg. with approxi-

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mately a 50% decrease in size of the thyroid glands. The thyroid of one dog sacrificed one hundred days after a similar dose showed fibrosis of vessels as well as the glandular tissue. No other tissues of any of these animals showed changes except the renal tubules of one of the rabbits.

Findlay and LeBlond (18) gave 60–80 $\mu\text{c.}$ of I^{131} to rats previously fed low iodine diets and found less marked histologic changes than those reported by Gorbman in his mice treated with 3 mc. per kg. These findings are consistent with the low dosage used.

If a direct comparison could be made between results in animals and in man, from 210 to 3,500 mc. would be required to produce total thyroid and parathyroid destruction in an average adult of 70 kg., or from ten to 175 times the usual maximum dose used in the treatment of Graves' disease. This safety factor is far greater than is usual with most therapeutic agents.

CALCULATION OF I^{131} DOSAGE

The subject of isotope dosimetry has been well covered by Marinelli (19), Quimby (20), and Marinelli, Quimby and Hine (21), the details of which are beyond the scope of this paper. It is of importance, however, to have some concept of the amount of radiation administered during the course of I^{131} therapy. Only then can conclusions be drawn as to the effectiveness of the method and comparisons between groups of patients be made.

I^{131} gives off two kinds of radiation, beta particles with maximum energy of 0.6 MEV (million electron volts) and gamma rays with energies of 0.37 and 0.08 MEV. Beta particles have a maximum range of approximately 2.5 mm. in tissue and most of their energy is thus expended within the thyroid. Gamma rays are more penetrating and produce but little radiation effect within the thyroid gland.

It is both simple and convenient to accept the following formula for the calculation of equivalent roentgens delivered to the thyroid by I^{131} (beta and gamma).

$$\text{Dose e.r.} = \frac{\mu\text{c. administered} \times \text{uptake \%}}{\text{grams of thyroid}} \times \frac{\text{Biol. H.L.}}{8} \times 160 \quad (15)$$

The dosage depends, then, upon the concentration of radioiodine within the thyroid, upon the half-life of the isotope, and upon the rate at which the radioiodine leaves the thyroid (the biological half-life). The figure "160" represents the approximate no. of equivalent roentgens produced when 1 $\mu\text{c.}$ of I^{131} undergoes total decay. The calculation of tissue dosage assumes that the distribution of the isotope within the thyroid is uniform (even though this is not always the case) and that each retained iodine

atom decays completely. The calculation of radiation dosage in this relatively easy way is of practical value even though it may lack absolute precision.

TECHNIQUE OF TREATMENT

I^{131} is administered orally. Single doses have ranged from 1.0 to 20.0 mc. with good therapeutic results with 2.0 to 12.0 mc. It is obvious that the amount of radioiodine taken up by the thyroid rather than the size of the oral dose is the important factor in determining the amount of radiation to the thyroid. Therefore, most workers have attempted to estimate the weight of the goiter, have measured the uptake of radioiodine by the thyroid gland and then have analyzed the therapeutic results in terms of the number of microcuries of radioactive iodine per gram of thyroid tissue. Haines and his coworkers (10) have devised a simple formula for the calculation of a desired dose:

$$\frac{\text{Microcuries desired per gram of tissue} \times \text{Estimated weight of thyroid in grams} \times 100}{\text{per cent of } \text{I}^{131} \text{ tracer collected by thyroid}}$$

This gives the total desired dose in microcuries.

The dose per gram of thyroid in the 83% of patients who have had good therapeutic results has been from 23.0 to 574.0 $\mu\text{c.}$ Werner, Quimby and Schmidt (11) state that doses of 100 $\mu\text{c.}$ per gram or more are generally adequate, since 24 of 27 patients returned to normal with a single dose of this size. However, no absolute rules can be made and the problem of dosage must ultimately fall upon sound clinical judgment rather than upon simplified formulations.

SELECTION OF PATIENTS

Probably because of the relatively short period of experience with radioiodine for therapeutic use the common denominator in the selection of patients seems to be the unquestioned diagnosis of toxic goiter. The Massachusetts General Hospital group prefer to limit their patients to those over 45 years of age since the possibility of late neoplastic change becomes more important in young patients.

Haines, Keating and coworkers (10) decided "to give therapeutic doses of radioiodine only to those persons for whom the risk and difficulties of other methods of treatment appeared to us (them) to be excessive" and included in their series patients with serious heart disease or other complications which added considerably to the surgical risk, and patients who had recurrent exophthalmic goiter, especially if the latter had had multiple resections of the thyroid and unilateral paralysis of a vocal cord or if extremely small amounts of thyroid gland remained. Their pa-

tients represent therefore ones whose disease had proved resistant to other forms of therapy.

Werner, Quimby and Schmidt (13) have selected patients as young as 18 years of age and have included in somewhat less than half of their series patients with hyperthyroidism, recurrent post-operatively, who had been treated with anti-thyroid drugs for some time without satisfactory relief. All but one of their patients had toxic diffuse goiter and none had true hyperophthalmic goiter.

Crile (16) has followed 22 of his 50 patients long enough to report results of radioiodine therapy. Of these 22, ten had toxic nodular and 12 toxic diffuse goiter. His patients with nodular goiter required larger doses and were more difficult to control than his other group. In addition, the nodular goiters did not decrease as readily in size.

The University of California group have used the following criteria in selecting patients for radioiodine therapy: (1) Definite primary hyperthyroidism up to moderately severe degree; (2) thyroid enlargement not much greater than 80 grams; (3) recurrent hyperthyroidism in patients who have had one or more thyroidectomies with or without parathyroid deficiency or recurrent laryngeal palsies and (4) severe exophthalmos when roentgen therapy seems inadvisable because of the large amount of thyroid tissue present. This group has considered certain factors contraindications to radioiodine therapy: (a) the presence of a nodular goiter; (b) extremely large thyroids with or without pressure symptoms; (c) severe thyrotoxicosis with complications since antithyroid drugs and other measures in conjunction with radioiodine probably provide a safer approach than radioiodine alone; (d) pregnancy; and (e) previous treatment with ordinary iodine unless there is adequate uptake of radioiodine by the thyroid. Prinzmetal, Agress, Bergman and Simkin (14, 15) also feel that patients with toxic nodular goiter should not be treated with radioiodine.

In summary, there are no hard and fast rules concerning selection of patients and many of the differences noted by groups of investigators are more apparent than real. Even after prolonged and extensive experience with any therapy in

any disease, there will be legitimate differences of opinion as to the best of several effective methods.

RESULTS OF TREATMENT OF GRAVES' DISEASE WITH RADIOIODINE I^{131}

The earliest published series of cases is that of Hertz and Roberts (7) who used I^{130} , with a half-life of 12.6 hours (see Table I). Chapman, Skanse and Evans (22) have also reported on the treatment of 65 cases of hyperthyroidism with I^{130} and their results are also included in Table I. In their group of 20 cases, Hertz and Roberts (7) followed radioiodine with a course of treatment with ordinary iodine (I^{127}). This was done so that no harm would result to the patient from prolonged thyrotoxicosis should the radioiodine prove ineffective in controlling the disease. It was thought also that the I^{127} might delay the release of radioiodine from the thyroid during treatment. In this respect, their series is not strictly comparable to that of Chapman, Skanse and Evans (22) who used I^{130} only but it appears likely from the published results that the major effects produced in those 20 cases were due to the radioactive

TABLE I
Treatment of Graves' disease with I^{130}

	I	II	III
	Hertz and Roberts (7)	Chapman and Evans (6)	Chapman, Skanse and Evans (22)
Number of patients	20	22	65*
Mean values			
Estimated wt. of thyroid before treatment	51.3 gm.	53.4 gm.	45 gm.
BMR before treatment	41.5%+	39%+	—
BMR after treatment	6.25%+†	1.4%+	—
Total dose I^{130}	10.8 mc.	—	32 mc.
Estimated dose in $\mu\text{c./gm.}$ of thyroid	—	860	450
Time to return to normal (mo.)	—	2½	2
Myxedema or hypothyroidism	—	2	11
Radiation sickness	—	3	6

* This group of 65 includes the 22 cases listed in column II.

† Done from 3 mo. to 4½ yrs. after treatment.

rather than to the inert iodine. Hertz and Roberts (7) reported on an additional nine cases in which I^{130} was not given with therapeutic intent and they, therefore, have been omitted from this series. The disadvantages in the use of I^{130} are the necessity for being near to its place of production (due to short half-life) and the more frequent production of radiation sickness because of rapid delivery of radiation and rapid destruction of thyroid tissue. It should be noted here that radioiodine which is produced by the cyclotron contains a mixture of isotopes, approximately 90% of which is I^{130} and 10% I^{131} ; thus much of the radiation effect could have been from the I^{131} .

When the longer-lived isotopes I^{131} (half-life, eight days) became more generally available, several groups began treating thyrotoxic patients in various parts of the country (8-11, 13-16, 22). A discussion of the use of this isotope is the primary aim of this paper. The results of therapy with I^{131} in some 288 cases are presented in summary form in Table II. It becomes apparent that considerable variation in methods and in results of treatment among the several groups occurs and this fact is worthy of some comment.

Haines, Keating *et al.* (10) at the Mayo Clinic in their treatment of more severe and complicated cases, used relatively large, single doses of I^{131} .

"A result was considered good if the basal metabolic rate was lowered to plus 15% or further and if the clinical evidences indicated that the patient was in a euthyroid state. Results were classified as fair when the severity of hyperthyroidism was markedly reduced by treatment." Twenty-seven of 40 patients had good results according to their criteria, eight were classified as fair results, and five were failures. Seven of 40 cases (17.5%) developed myxedema following treatment. In view of the nature of the patients treated it obviously was better to overshoot than to undershoot the mark in therapy.

Werner, Quimby and Schmidt (13) at Columbia University reported upon the results of treatment of 58 patients with I^{131} with follow-up studies of from four months to over one year (not included in Table II because of incomplete published data). The average total dosage used was from 3 to 4 mc. with 22 of the 58 patients requiring more than a single therapeutic dose. Forty-eight of 56 patients are now in remission, a case success rate of 86%. Two patients have not been followed sufficiently since the last treatment to permit evaluation. Four cases became hypothyroid for a short period of time but recovered. The average time required to reach normal metabolic levels following treatment was three months. Nearly 50% of

TABLE II
Treatment of Graves' disease with radioiodine (I^{131})

	I Chapman, Evans and Skanse	II Soley and Miller		III Haines, Keating <i>et al.</i>	IV Crile		V Prinzmetal <i>et al.</i>	
		<i>good results</i>	<i>fair results</i>		<i>diffuse</i>	<i>tox. nodular</i>	<i>uncomplic.</i>	<i>complic.</i>
Number of patients	65	51	10	40	12	10	26	18
<i>Mean values</i>								
Est. wt. of thyroid before treatment (gm.)	50 gm.	31.4	49.1	27.6	61	115	38.7	42.3
Est. wt. of thyroid after treatment	—	13.1	19.0	smaller	28	80	20	20(?)
BMR before treatment	—	28.3+	39+	35+	47+	48+	—	37.5+
BMR after treatment	—	1—	6.8+	5.7+	2+	24+	—	0
Prot-bound iodine before treatment (μc. %)	—	10.7	13.7	—	—	—	11.3	11.5
Prot-bound iodine after treatment	—	5.8	7.7	—	—	—	6.3	6.3
Total dose I^{131} (μc.)	10,000*	3,067	7,556	—	7,200	10,900	4,400	5,320
Estimated μc./gm. of thyroid for total dose	140	67	162	230.65*	—	—	—	—
		(42 cases)	(4 cases)					
Time until normal (mo.)	2	3.4	14.6	2.67	4	0	3-4	4.1 (17 cases)
Myxedema and hypothyroidism after treatment	4	3	0	7	1	0	4	0
Radiation sickness	0	0	0	1?	0	0	0	0
Relapses	0	1	0	3	0	0	1	0
Classification of results								
Good	56/65-86%	51/61-84%		27/40-67.5%	11/12-91.5%	4/10-40%	25/26-96%	16/18-89%
Fair	8/65-12.3%	10/61-16%		8/40-20%	1/12- 8.5%	6/10-60%	1/26- 4%	2/18-11%
Poor		(2 failures) (not included in above)		5/40-12.5%				

* Single dose.

their patients represented recurrent hyperthyroidism after operation; they were not considered to be good surgical risks, and, for the most part, had not come into remission with antithyroid drugs or with X-ray therapy.

Sixty-one patients have been treated at the University of California Hospital (8, 9). Each of the patients treated had either primary or recurrent Graves' disease of mild to moderately severe degree. When the percentage uptake of iodine by the thyroid had been determined, it was possible to make an estimate of the probable optimal dosage of iodine needed, since it could be shown that, over a short period of time, the uptake of a large (therapeutic) dose as a rule would be the same as that of a small (tracer) dose. Following treatment, patients were re-evaluated at two to four week intervals and second, third, or fourth treatments were given as necessary. The usual interval between treatments was from four to six weeks. A good result was one in which complete remission of the signs and symptoms of Graves' disease occurred with return of the thyroid to normal size within a reasonable length of time, irrespective of the number of doses required. Of the 61 cases which now have been reported, 51, or 84%, were considered to be good results. The remaining ten patients (16%) were reported as fair results due to the fact that they were under treatment for a long period of time before reaching the euthyroid state. It is apparent that these ten patients represent, on the average, a more severe degree of hyperthyroidism and that larger doses of radioiodine were necessary for the control of their disease. At least a part of the difficulty lay in the fact that $\frac{1}{10}$ of these patients were treated rather early in the course of this study when only limited amounts of iodine were available and when low dosage schedules were being used. Three patients could not be followed or evaluated properly and represent therapeutic failures. The first was a 35 year old woman with severe Graves' disease. She received a total of 4 mc. of I^{131} in two doses, with return to normal thyroid status within two months of the beginning of therapy. Two months later she developed an acute rheumatic pancarditis and it became impossible to appraise her thyroid status accurately. The second case was a 53 year old woman with symptoms and signs of hyperthyroidism, with goiter, elevated BMR and high protein-bound

iodine. She was given a total of 6 mc. of I^{131} without improvement in her clinical condition. She later underwent a subtotal thyroidectomy at another hospital and the thyroid was found to be nodular and fibrotic. The third failure was a woman whose treatment for Graves' disease with radioiodine was interrupted by the development of a major psychotic episode. The latter two patients were not included in the tabulated results.

The 65 cases reported by Chapman, Skanse, and Evans (22) of the Massachusetts General Hospital are quite comparable with those from the University of California. Although the average total dose of radioiodine (in millicuries) exceeds that given the University of California group and was administered as a single dose, the estimated number of microcuries per gram of thyroid is quite similar, as are the results of treatment.³ Fifty-six of the 65 patients experienced good results following I^{131} therapy whereas eight of 65 were improved but not clinically well six months after treatment. Four of their cases developed hypothyroidism but there were no other complications. One of the 65 died of myocardial infarction shortly after treatment and therefore is omitted from the tabulated results.

Crile (16), at the Cleveland Clinic, has treated 50 patients with radioiodine and has reported upon the results in 22 cases who have been followed for from three to ten months. Twelve patients had diffuse toxic goiter and the remaining ten toxic nodular goiter. Eleven of the 12 patients in the first group responded well to treatment, with relief of symptoms of hyperthyroidism and a significant decrease in thyroid size. Only one patient in this group was not classified as a good result even though much improved. Because of the relatively short period of follow-up, it is perhaps not justifiable to class this as a poor result. The patients with toxic nodular goiter have proven considerably more difficult to control by this method of therapy; they require larger doses of radioiodine to produce a remission in their disease, the length of time required to bring about improvement is longer,

³ At the time that these studies were carried out, the millicurie as measured at the University of California apparently was about $2\frac{1}{2}$ times that of the Massachusetts General Hospital; consequently the average total dosage is more nearly the same than would appear from the data.

and the nodular thyroid does not decrease as readily in size. Of ten such cases, only four (40%) have made a clinical recovery while the remaining six are still under treatment. (Results tabulated in Table II.)

Prinzmetal and co-workers (14, 15) have compiled complete data on 44 patients, all of which have been followed for more than one year. Twenty-six were uncomplicated cases of Graves' disease, comparable with those of the University of California group. Using a schedule of multiple, relatively conservative doses, Prinzmetal succeeded in producing satisfactory remissions in 25 of 26 patients within three to four months. The last patient of the group is reported to be improved, but not completely well after one year of study. "Three patients developed hypometabolism and one patient developed signs and symptoms of myxedema. One patient who apparently relapsed had complete return to normal after further iodine administration." The remaining 18 cases presented one or more rather serious complications in addition to their hyperthyroidism. Of the group, 16 (89%) became relieved of their thyrotoxicosis within four months, on the average. One patient did not respond at all and another was improved only moderately.

The overall incidence of clinically good results in the 288 hyperthyroid patients summarized is 83%; 9.7% can be classed as fair results; 5.5% as failures. Results of treatment of toxic nodular goiter with radioiodine have been relatively disappointing since only four out of ten did well.

Some attempt should be made to explain the differences in results obtained in patients when equal quantities of radioiodine are given, and in various series of patients who presumably are handled in the same way. Probably not a great source of discrepancy is the variability of standardization of the iodine at different institutions throughout the country. The major difficulty lies in the calculation of radiation dosage for any given patient. Apart from the factors of physical characteristics of the administered radiation, dosage is dependent upon (1) the percentage which is taken up and held by the thyroid, (2) the size of the gland in which the iodine is distributed, and (3) the rate at which the iodine leaves the thyroid. The greatest source of error is, of course, in the estimation of thyroid size, a subjective error which cannot

readily be measured. Even if it were possible to guess accurately the weight of the thyroid, certain differences in therapeutic results would be observed due to variations in response to a given amount of radiation in individual patients. Hence, as has been reported by several groups (8-13), myxedema may follow relatively small doses of radioiodine (in $\mu\text{c.}$ per gram) while patients receiving much greater amounts do not go below normal metabolic levels.

COMPLICATIONS

Certain phenomena seen within the immediate post-therapy period are worthy of comment but do not constitute true complications. Within the first two to three days, spontaneous tenderness in the thyroid or tenderness on palpation may be noted and this coincides with an increase in the sedimentation rate. This is seen mainly after single doses of two or more millicuries of I^{131} .

Within the first one or two weeks after I^{131} has been administered, some increase in the severity of thyrotoxic symptoms may occur and is associated with a rise in the protein-bound iodine in the blood. Werner, Quimby and Schmidt (11) state that the average increase in the basal metabolic rate in three patients was 15%. The mechanism of the exacerbation probably is rapid radiation destruction of the thyroid and release of stored hormone.

Patients and examiners alike are apt to find that the thyroid becomes firm and even hard within a few days after therapy. This may be associated with a sore throat and cough, the latter persisting for several weeks.

In only one instance has radiation sickness been noted (10), and this was questionable. This is in distinct contrast with the findings of Chapman and Evans (6) who observed radiation sickness in six of 65 patients treated with I^{130} .

Transient hypothyroidism has been seen in four of 58 patients (13), actual hypothyroidism in three of 26 patients (14, 15) and myxedema in 16 of the entire 288 patients treated with I^{131} .

Five deaths occurred among the 288 patients within a period of days to several months following treatment—a remarkable record when one considers the severity of illness of some of the patients treated especially by Prinzmetal's and Haines' groups. Two of the five died of myo-

cardial infarction, two of cerebral-vascular accidents and one of cardiac failure.

Apparently, severe progression of exophthalmos has not been a troublesome feature of radioiodine therapy. Soley, Miller, and Foreman (9) found a progression of 1.5 mm. in 11 of 50 patients and an increase of 3 or more mm. in four of the 50 while none showed a decrease of 1.5 mm. or more. Thus 30% showed a significant increase in the prominence of their eyes. By way of comparison, 26% of 75 hyperthyroid patients who received roentgen-ray therapy had progression of 1.5 mm. or more and 6.6% showed a decrease of the same magnitude. Following subtotal thyroidectomy for Graves' disease, 40-50% of the patients had an increase in the prominence of their eyes of 1.5 mm. or more.

Relapses after probably inadequate therapy have occurred in the experience of all investigators but true recurrences are rare, because the length of follow-up has not been of sufficient duration for recurrence to take place in well-treated patients.

COMPARISON WITH OTHER METHODS OF TREATMENT

Means (23) in 1946 appraised the modern methods of treating Graves' disease but did not have the advantage of recent published work with antithyroid drugs or radioactive iodine. Up to the time of writing this paper, data still are not available to determine precisely the relative merits of (1) preparation of the patient with an antithyroid drug followed by subtotal thyroidectomy, (2) treatment with antithyroid drugs alone, (3) roentgen-ray treatment, and (4) treatment with radioiodine.

These various methods may be evaluated briefly as follows:

(1). In skilled hands with an efficient team, subtotal thyroidectomy in the well-prepared patient who has been on propyl thiouracil or one of the other thiouracil derivatives, carries a mortality of 0.5% or less and is accompanied by the hazards of parathyroid and recurrent laryngeal nerve injury in a small percentage of patients. In addition a varying number of patients will develop myxedema which can be treated readily with thyroid. The recurrence rate is under 5%. Probably more patients can be treated successfully this way than by any single method.

(2). The commonly used thiouracils will in nearly all patients produce a satisfactory remission in Graves' disease. Thiouracil carries the same mortality hazard as subtotal thyroidectomy and in turn produces toxic effects in nearly 15% of patients, the most serious of which is agranulocytosis in approximately 2%. Propyl thiouracil is much safer. However, after six to nine months of full remission, from perhaps 15% to over 50% of people will have a recurrence of hyperthyroidism when the drug is discontinued. The danger of neoplastic changes remains an unknown quantity. Advantages are the simplicity of therapy, the general satisfactory response of the patient and relatively low cost.

(3). Roentgen-ray therapy is not widely employed by thyroidologists, but has given good therapeutic responses in selected patients: those with moderate hyperthyroidism, with non-nodular goiters up to about 60 grams without complications such as coincident heart disease in which a rather slow return to normal might jeopardize the patient and with no history of sun-sensitivity or chronic laryngitis. The chief disadvantage is the comparatively long period required for complete remission. A great advantage is the absence of any mortality from the therapy itself.

(4). Radioiodine can be expected to produce satisfactory remissions in over 80% of patients and many of the other patients would (with less strict criteria than justifiably conservative observers have established) have had reasonably satisfactory end results. The only recorded deaths following I^{131} were not related to the iodine therapy. No serious complications have been noted, but the possibility of late radiation change (tumors) has not been excluded. A disadvantage is the need for technical equipment and skilled personnel beyond that required for other methods of therapy.

Probably the most logical attitude for the therapist in Graves' disease is the recognition of at least four empirical but satisfactory types of treatment, any one of which or combinations of which should be selected for a given patient depending upon individual consideration of the patient and the special skills of the physician.

SUMMARY

An attempt has been made to present the total experience, to date, in the treatment of thyrotoxi-

cosis with radioactive iodine. Although there are differences among various groups in the methods of treatment, the end results are quite similar and compare favorably with already accepted forms of therapy.

The use of radioactive materials in clinical medicine brings with it the necessity for specially trained personnel, and strict attention to the potential hazards of radiation both to patients and to physicians. In this, the relatively young era of isotope application, progress must be made slowly and carefully. It is not unlikely, however, that radioiodine therapy ultimately will come into more general use.

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THE UPTAKE AND ELIMINATION OF KRYPTON AND OTHER INERT GASES BY THE HUMAN BODY¹

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INTRODUCTION

Chemically inert gases, such as nitrogen, helium, neon, argon, krypton and xenon, apparently do not participate at normal pressures in biochemical reactions of the human body. These gases are present in physical solution, chiefly in the body water and fat. In recent years much interest has been focused on the exchange of these gases between body fluids and external air, through the lungs, skin and intestinal wall. A number of important physiological processes may be studied by means of inert gas exchange measurements.

During rapid decompression from several atmospheres to one atmosphere or from one atmosphere to a fraction of an atmosphere the dissolved inert gases originally in equilibrium may become relatively supersaturated so that under certain conditions gas bubbles may form in the blood and tissues (1). These may exert mechanical pressure on nerve endings or may cause pain by some other mechanism (2). Our investigations were initiated with the explicit desire to provide (a) classification procedures for selection of high altitude flyers on the basis of gas exchange rates; (b) information on methods of prevention of bends by accelerating the elimination of nitrogen or inert gas or by pre-breathing oxygen. In attempting to solve these two problems, which were of immediate practical importance, we could spend relatively little time on the study of the fundamental mechanisms of inert gas exchange. Answers to the problems *a* and *b* have been given elsewhere (3, 4) and the purpose of this paper is to describe some experiments which pertain to the mechanism of inert gas exchange in the human body.

After Zuntz (5) postulated a mechanism for the exchange of dissolved nitrogen between the tissues and the lungs, Boycott *et al.* (6) carried out experiments on goats and men subjected to excess pressure and determined the general shape of the nitrogen desaturation curve. Bornstein (7) and later Campbell and Hill (8, 9) made further studies of nitrogen exchange, showing that rates of exchange are different in various parts of the body. Shaw *et al.* (10) demonstrated on dogs that under conditions of equilibrium at pressures up to four atmospheres, the nitrogen content of the body is proportional to the partial pressure of nitrogen in the lungs, and that the nitrogen saturation time is the same as the desaturation time. Behnke *et al.* (11-14) also showed that the slope of the nitrogen elimination curve is a function of the cardiac output and suggested that helium exchange is faster, argon exchange slower than that of nitrogen, gas elimination more rapid from the fluid constituents of tissues, and slower from tissues high in fat, and showed that helium and nitrogen may diffuse slowly through the skin. Throughout most of the above described work the whole body elimination was studied more thoroughly than that of specific regions because of lack of suitable methods. Recently Ferris *et al.* (15) measured the nitrogen exchange in man by determining changes in nitrogen content of arterial and venous blood samples. They found that arterial blood reaches equilibrium with pulmonary nitrogen within a few minutes, while venous blood reaches equilibrium much more slowly. Whiteley *et al.* (16) observed the same in the femoral vessels of cats and found that exercise increases the rate of denitrogenation. During the war Smith and Morales also carried out some similar experiments (17-19). The mechanism of total body gas exchange was put on a new basis as a result of work by Jones and his collaborators (20) who demonstrated that diffusion plays very little role in the uptake of various gases by tissues, and that the rapidity of gas exchange depends mainly on the gas carrying power

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TABLE I
 Properties of the radioactive inert gases suitable for biological investigations *

Name	Isotope	Type of radiation	Energy of radiation		Half life	Most important nuclear reaction
			Particles mev	γ rays		
Nitrogen	13	β^+	1.24	no γ	9.93 minutes	$C^{12}(d, n)$
Argon	37	K	—	no γ	34 days	$Cl^{37}(d, 2n)$
Argon	41	β^-, γ	1.5	.37	110 minutes	$A^{40}(d, p)$
Krypton	79, 81	β^+, γ	$\sim .9$ (30%); 0.6 (70%)	0.2	34 hrs.	$Br^{79,81}(d, 2n)$
Krypton	85	β^-	0.74	no γ	9.4 yrs.	Kr (n, γ)
Xenon	127	e^-, γ	—	0.9	34 days	U-n
Xenon	133	β^-, γ, e^-	0.42	0.085	5.3 days	$I^{127}(d, 2n)$
Radon	222	α	5.486	—†	3.825 days	U-n

* For references and details see G. T. Seaborg and I. Perlman, Table of isotopes. Rev. Mod. Phys., 1948, 20, 585. Also see Way *et al.*, Nucleonics, 2, No. 5, Part 2, May, 1948.

† γ rays from radioactive daughters.

of the blood, that is gas solubility in blood and the rate of perfusion of the tissues by blood.

EXPERIMENTAL METHODS

A glance at Table I shows that it is difficult to work with radioactive nitrogen because of its short half life—the gases argon, krypton and xenon are much better suited for tracer investigations. Fortunately it is permissible to use these gases instead of nitrogen if suitable correction factors are applied in interpreting the results. Two kinds of corrections are needed. First one has to take account of the fact that the water and fat solubilities of these gases are different. A detailed analysis of these solubilities by Lawrence *et al.* (21) is summarized in Table II. In addition, another correction factor must be used whenever diffusion of these gases influences the rate of gas exchange. As predicted by Graham's Law, Jones *et al.* recently demonstrated that the noble gases diffuse through gelatin membranes at room temperature at rates which vary inversely with the square root of the molecular weights of the gases.

In the present series of tests the absolute amount of inert gases in the tissues was emphasized less than the relative rate of their uptake and desaturation. Prior to

uptake measurements the radioactive gases were introduced into a closed circuit spirometer. The concentration of gas in the spirometer was determined by a Geiger-Mueller counter. In most experiments this concentration was held constant throughout the period of radioactive gas uptake in the body. In this way the final volume of the spirometer did not enter into consideration when the rate of body uptake was determined. The simple method of measurement required placing a Geiger-Mueller counter against the body region studied and continuously recording the counting rate.³ The schematic setup is shown in Figure 1, Diagram 1a. A counter was held gently in one hand, surrounded by a lead shield, as shown in Figure 1, Diagram 1c, and the left knee was placed on a lead slit, limiting the solid angle of measurement for another counter (Figure 1, Diagram 1b).

Preparation of the radioactive gases in the cyclotron required special techniques. Except in the cases of N^{13} , and A^{41} , halides were bombarded by deuterons; potassium chloride for A^{37} , potassium bromide for $Kr^{81, 79}$ and potassium iodide for Xe^{127} . Because of the high intensity of the deuteron beam, it was necessary to design a target which spread the deuteron beam over a relatively large area so that heat generated could be dissipated. The target plate was tilted so that the effective portion of the beam was spread over an area of 30 square cm. It was also found necessary to isolate the space above the target from the cyclotron vacuum because during bombardment a considerable amount of occluded gas may be released from the target and this would seriously hamper the maintenance of a proper degree of vacuum in the cyclotron as well as cause a loss of the radioactive noble gas formed during the period of exposure. Two thin aluminum windows, cooled by compressed air, were used. The space above the target (the so-called bell jar) was usually filled with He at $\frac{1}{2}$ atmospheric pressure. The target plates themselves were made of $\frac{1}{4}$ inch copper plate with a grooved surface. The halide salts were fused to the reduced surface of the target at a

³ These methods were developed in collaboration with J. B. Mohny and F. W. Loomis of this laboratory.

 TABLE II
 Solubilities of inert gases in water and oils at 37° C.

Gas	Bunsen's absorption coefficient (two significant figures)			
	Molecular weight	Water	Olive oil	Oil/water solubility ratio
Helium	4	0.0085	0.015	1.7
Neon	20.2	0.0097	—	—
Nitrogen	28	0.013	.067	5.2
Argon	39.9	0.026	.14	5.3
Krypton	83.7	0.045	.43	9.6
Xenon	131.3	0.085	1.7	20.0
Radon	222	0.15	19.0	125.0

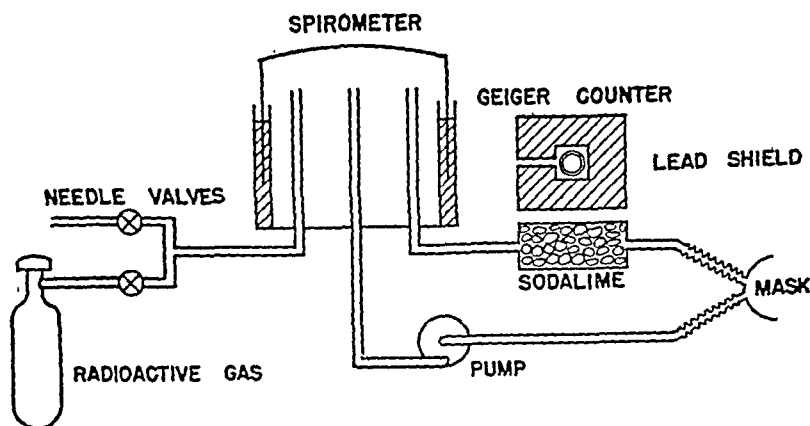


DIAGRAM 1a

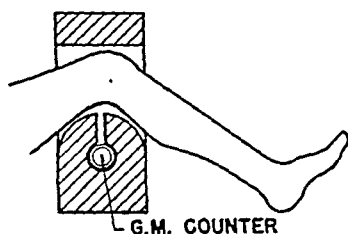
LEAD SHIELD FOR
MEASUREMENT OF ACTIVITY
IN THE KNEE REGION

DIAGRAM 1b

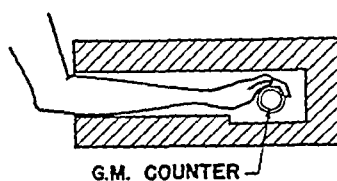
LEAD SHIELD FOR
MEASUREMENT OF ACTIVITY
IN THE HAND REGION

DIAGRAM 1c

FIG. 1. SCHEMATIC VIEW OF THE APPARATUS USED FOR RADIOACTIVE GAS EXCHANGE MEASUREMENTS

Diagram 1 a. Spirometer, mask and gas recirculating system.

Diagram 1 b. Method of measuring radioactivity of the knee region.

Diagram 1 c. Method of measuring radioactivity in the hand region.

temperature of approximately 650° C. In addition to potassium, halides of Rb, Sc, Sr and Ba have also been employed with no advantage over potassium salts. Lithium halides are undesirable because of their low melting points and hygroscopic properties, and sodium salts are not used because of the very intense gamma ray activity arising from Na^{24} after bombardment. About 10% of the radioactive inert gases was collected in the bell jar. The remainder is retained in the salt. The bombarded salt is usually scraped off the target plate and fused in a sealed evacuated quartz test tube. The occluded gas is easily driven off at a temperature of 650° C. along with some halogen vapor contamination, usually radioactive (e.g., Br^{80} , 4.4 hours; Br^{82} , 34 hours; I^{129} , 25 minutes). These vapors are absorbed when passing through alkaline sodium sulfide solution. The radioactive inert gas along with some inactive helium and nitrogen is then collected in an evacuated bulb. In most experiments 1–2 cm³ of carrier gas was added, then the sample was compressed in a suitable bottle with pure oxygen to ~250 lbs/sq. inch. Calibration of the radioactivity was accomplished by taking a known volume of mixed gas from the compressed sample and determin-

ing its γ activity in comparing it with a radium standard. During experimental use the gas flow was regulated by a needle valve, and the volume measured by a spirometer (see Figure 1). It is possible to salvage some of the long-life radioactive gases after each experiment for repeated use by collecting the entire expired air during the test, then re-absorbing it in silica gel at liquid air temperature. Recovery of the gases from the silica gel is carried out in the following manner. The silica gel is slowly heated, oxygen, nitrogen, and carbon dioxide are liberated first, followed by radioxenon or krypton. About 60% of the original radioxenon or krypton can be recovered by this method in the last liter of gas given off. Purification processes for somewhat different purposes are also described by Brown *et al.* (22).

The amount of radioactivity necessary for each uptake desaturation experiment was about 0.4 millicurie⁴ and less than half this amount was absorbed in the body.

⁴ Since this work was done, the potential efficiency of gamma ray counting has been greatly increased by the use of fluorescence counters. This will allow the use of smaller radioactive samples in such experiments in the future.

All data were corrected for radioactive decay by multiplying each counting rate by $e^{\lambda t}$ where λ is the decay constant of the radioactive gas used; t is the time elapsed since the beginning of the experiment.

EXPERIMENTAL RESULTS

The relative uptake and desaturation curves of the right hand and left knee were obtained by measuring the rate of gamma ray counts from them in function of time. A typical result obtained in two different experiments on the same person is shown in Figure 2. The counting rate was 3000/minute near the peak of the curve. The experimental points did not fall exactly on the interpolated curves, due to random statistical fluctuations in the number of quanta emitted. If the subject moved during the experiment, other deviations in the measured counting rate occurred. For these reasons exact mathematical analysis of the data is not possible. At the beginning of the uptake experiments there usually was a delay between the time when breathing from the mask started and the time the first traces of radioactive gas appeared in the extremities. The delay amounted to 30 to 90 seconds and was due to the time required for mixing of the gases of alveolar

air and spirometer (20 to 40 seconds half time) and transportation of the blood to the extremities. In searching for a suitable analytical expression for the shape of the uptake and desaturation curves we attempted to divide them into sums of simple exponential functions. The hand data could be satisfactorily approximated by the sum of three exponential functions. Analysis of the knee data makes it appear that the fastest of the three components has a negligible contribution so that the knee data, as well as total uptake data, may be described as the sum of two exponential functions of time. Unfortunately complete saturation of the hands or knees was not reached in the three hours maximum period of the "uptake" experiment. Because of discomfort of the subjects breathing the radioactive gas mixture from the somewhat uncomfortable masks, no attempts were made to follow the uptake longer than three hours. The desaturation, with the subject breathing air in a well ventilated room, was carried out up to 12 hours.

Four subjects participated in two consecutive tests each; in one test they inhaled radiokrypton at a constant concentration for a short period of

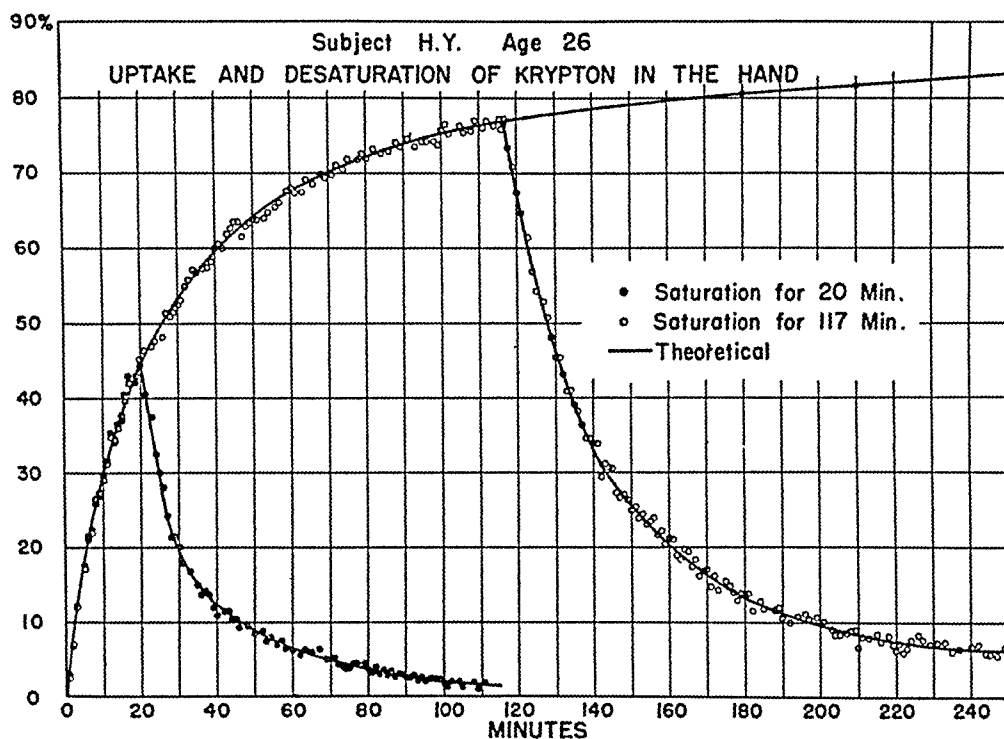


FIG. 2. TYPICAL UPTAKE AND DESATURATION EXPERIMENTS WITH RADIOKRYPTON

The concentration of radioactive gas in the spirometer was held constant. The theoretical curves represent the sum of three exponents adjusted to the data as described in the text.

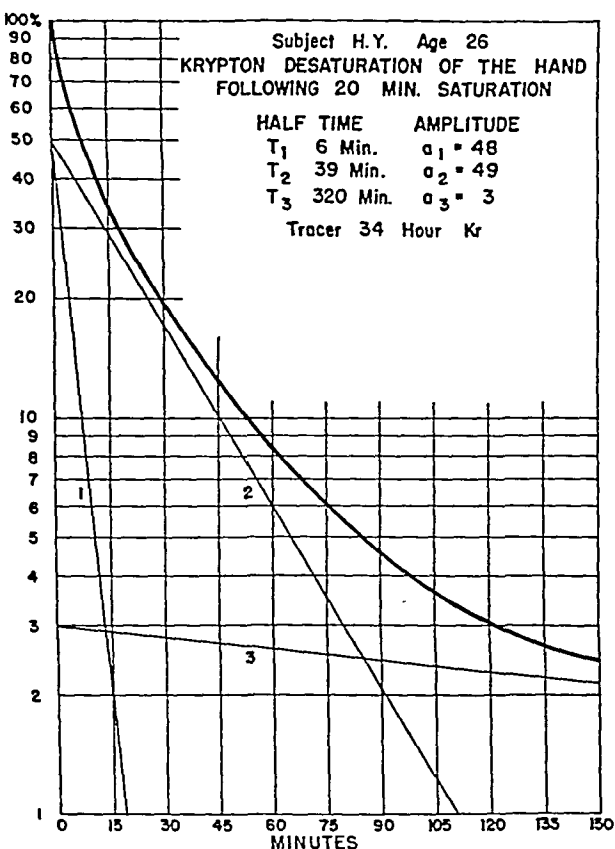


FIG. 3. PLOT OF DESATURATION CURVE ON SEMI-LOGARITHMIC PAPER AFTER SATURATION OF SUBJECT WITH RADIOKRYPTON FOR 20 MINUTES

The three components of desaturation are represented by straight lines.

time (about 30 minutes); the mask was then taken off and desaturation was continued by breathing air. In the second test, uptake was continued for about 120 minutes, followed by desaturation up to 12 hours.

Figure 2 shows the shape of the hand uptake and desaturation curves in two different experiments on the same subject. The uptake Φ in this and other experiments was found to fit the empirical formula:

$$\Phi = A_1(1 - 2^{-(t/T_1)}) + A_2(1 - 2^{-(t/T_2)}) + A_3(1 - 2^{-(t/T_3)})$$

is the sum of three exponential type "saturation" curves. The "amplitudes" of these three components are A_1 , A_2 and A_3 and the "half saturation times" characteristic of the rate of krypton uptake are T_1 , T_2 and T_3 ; while t is the time elapsed after the beginning of the gas uptake. The data are plotted in such a way that

$$A_1 + A_2 + A_3 = 1.$$

The shape of the desaturation curves differs from the saturation curves, inasmuch as the desaturation after 20 minutes uptake does not follow exactly the same law as desaturation after 120 minutes uptake. To obtain some information regarding the analytical shape of the desaturation curves, they were plotted again in Figures 3-5 on semilogarithmic scale. It became clear the desaturation curves Ψ may be expressed approximately as sums of three exponentially decaying functions of the form

$$\Psi(\kappa, \tau) = a_1 2^{-(\tau/T_{d1})} + a_2 2^{-(\tau/T_{d2})} + a_3 2^{-(\tau/T_{d3})}.$$

Here κ is the time taken for saturation; a_1 , a_2 , and a_3 are amplitudes again; τ is the time elapsed since beginning of desaturation; T_{d1} , T_{d2} , T_{d3} are characteristic desaturation half times. The measured values of some of the constants " a " and T_d are given in Table III. It is found by inspection of Table III, and similar data taken on other subjects, that the values T_d are about the same irrespective of the length of time of uptake, while the amplitudes " a " are different for the two different experiments.

Comparing the constants of the uptake and desaturation experiments it was found that within reasonable agreement of the data obtained the constants expressing

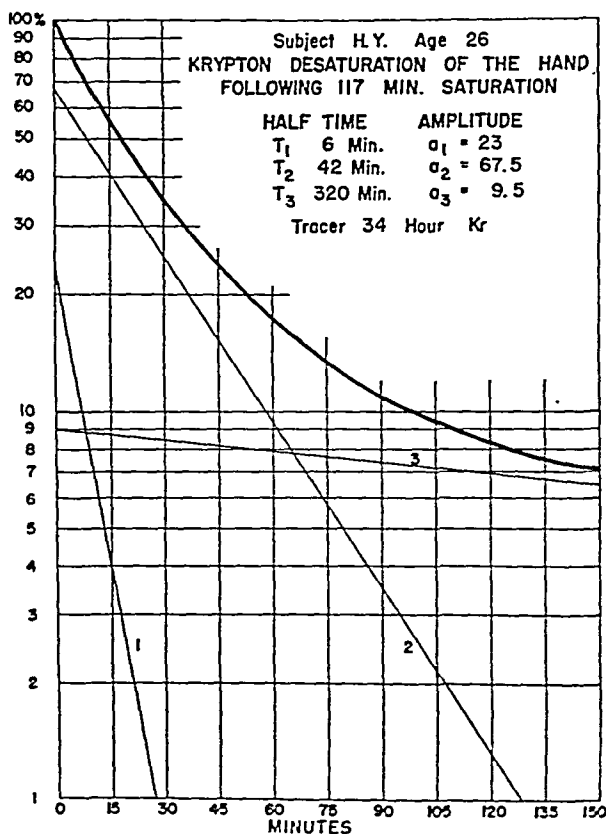


FIG. 4. PLOT OF DESATURATION CURVE ON SEMILOGARITHMIC PAPER AFTER SATURATION OF SUBJECT WITH RADIOKRYPTON FOR 117 MINUTES

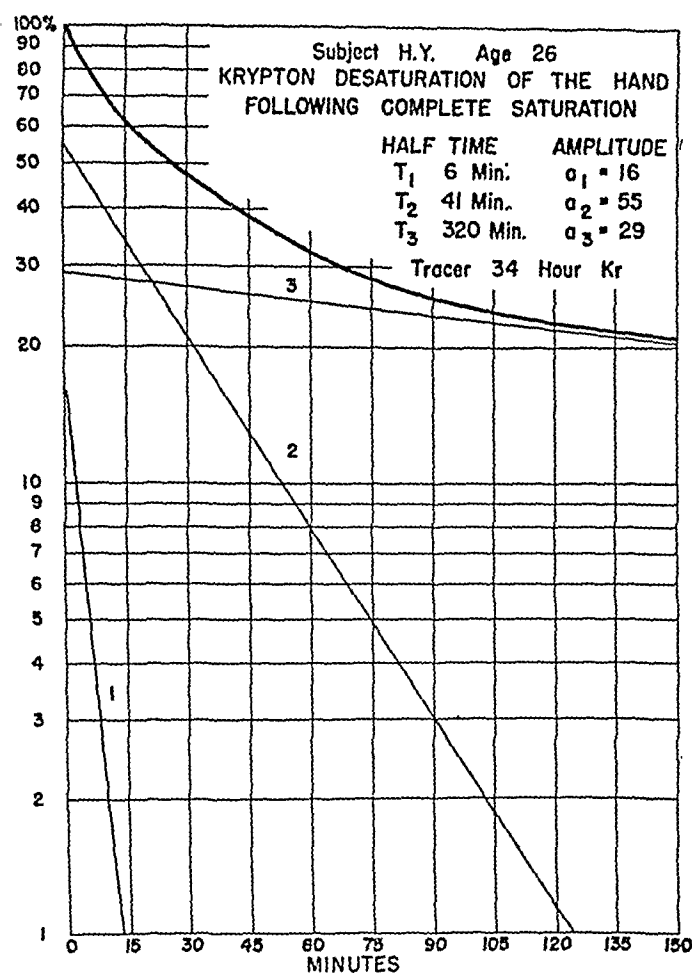


FIG. 5. PLOT OF DESATURATION CURVE ON SEMI-LOGARITHMIC PAPER AFTER COMPLETE SATURATION OF SUBJECT WITH RADIOKRYPTON

the empirical uptake curve and desaturation curve are related. First the half saturation and half desaturation times are about equal

$$\begin{aligned} T_1 &\approx T_{d1}, \\ T_2 &\approx T_{d2}, \\ T_3 &\approx T_{d3}. \end{aligned}$$

Second, the amplitudes "a" of desaturation curves, obtained after saturation for time κ obey the following approximate relationships:

$$\begin{aligned} a_1 &= A_1(1 - 2^{-(\kappa/T_1)}), \\ a_2 &= A_2(1 - 2^{-(\kappa/T_2)}), \\ a_3 &= A_3(1 - 2^{-(\kappa/T_3)}). \end{aligned}$$

Consequently, using the constants obtained in uptake experiments, the empirical formula of desaturation after saturation may be obtained as

$$\Psi(\kappa, \tau) = A_1(1 - 2^{-(\kappa/T_1)})2^{-(\tau/T_1)} + A_2(1 - 2^{-(\kappa/T_2)})2^{-(\tau/T_2)} + A_3(1 - 2^{-(\kappa/T_3)})2^{-(\tau/T_3)}.$$

Data obtained on four different subjects bearing on this point are given in Table IV. Further analysis of the data are possible along the lines of thought of Smith and Morales (17-19) or Tobias (23). These might result in better knowledge of the transport mechanisms involved.

TABLE III

Amplitudes and half desaturation times of the same subject after different saturation times

Half desaturation times τ			Amplitudes a		
	τ after 20 minutes uptake	τ after 117 minutes uptake		After 20 minutes uptake	After 117 minutes uptake
	minutes	minutes			
T_1	6	6	a_1	.48	.23
T_2	39	42	a_2	.49	.675
T_3	310	320	a_3	.03	.095

It would appear then that saturation of the extremities with inert gases is not a strictly reversible phenomenon with desaturation as far as time dependence of the process goes. One may say that it appears as though in the hand there were at least three distinctly different reservoirs containing inert gas; they may be distinguished by the rapidity of the dissolved gas exchange. The filling of these three reservoirs is somewhat independent from each other inasmuch as the time of filling, as characterized by the half saturation time, falls in a definite range. The saturation of each reservoir may be at the present state of experimental accuracy, expressed by a single exponential type function.

TABLE IV
Constants of uptake

		A_1 or a_1	T_1 min.	A_2 and a_2	T_2 min.	A_3 and a_3	T_3 min.
H. Y. 26	Uptake (117 minutes)	.16	6	.55	42	.29	320
	Desaturation after 20 minutes uptake	.49	6	.48	39	.03	320
	Desaturation after 117 minutes uptake	.23	6	.67	42	.10	320
R. C. 24	Uptake (90 minutes)	.05	5	.40	39	.55	140
	Desaturation after 20 minutes uptake	—	—	.73	16	.17	140
	Desaturation after 90 minutes uptake	.09	5	.56	39	.35	140
C. T. 25	Uptake 165 minutes	.10	2	.68	40	.22	315
	Desaturation after 30 minutes uptake	.20	2	.76	17	.04	366
	Desaturation after 165 minutes uptake	.12	2	.78	40	.10	315
E. F. 21	Uptake 155 minutes	.14	8	.67	50	.19	250
	Desaturation after 102 minutes uptake	.27	5	.68	47	.05	267
	Desaturation after 155 minutes uptake	.18	8	.73	50	.09	250

TABLE V
Uptake of krypton gas in the hand

Name	Age	First component		Second component		Third component		Bends susceptibility
		Amplitude	Half time	A ₂	T ₂	A ₃	T ₃	
			minutes		minutes		minutes	
M. C.	17	.19	3	.66	32	.15	100	r
E. W.	18	.13	6	.59	35	.28	145	r
G. B.	20	.17	5	.61	40	.22	129	s
E. F.	21	.14	8	.66	50	.20	315	s
S. C.	21	.06	4	.65	32	.29	104	s
D. D.	21	—	—	.14	13	.86	125	r
R. G.	24	.05	5	.40	39	.55	140	s
C. T.	25	.10	2	.68	40	.22	315	i
H. Y.	26	.16	6	.55	42	.29	320	—
Average	21.4	.11	4.3	.54	33	.35	188	

r = relatively resistant. s = susceptible. i = intermediate.

Examining the three components in a typical uptake curve one might say that the fastest component probably has something to do with the inert gas exchange between blood and very vascularized tissues. The $\frac{1}{2}$ saturation time (T_1) for this component appears to be ~ two to 10 minutes for the hand. This component of the hand curve accounts in intensity for 2 to 15% of the total radioactive gas content. Its rapidity is in good agreement with the direct findings of Cook and Sears (24) on dogs, and Ferris *et al.* (15) for humans. They find that exchange of inert gases (krypton in dogs, nitrogen in humans) with arterial blood is rapid; one passage of blood through the lungs is enough to empty or fill it with gases to ~ 80 to 95% of the equilibrium value. After a few seconds of transport time the arterial blood enters the hand; in its passage through the capillaries, it uploads most of the radioactive gas to the tissues and returns as venous blood empty of gas to the lungs where it takes up more. The characteristic of this component is its variability in half saturation time, or amplitude; this is to be expected and further data regarding it will be presented below. In the knee region this component may be found only with a very small amplitude: the amount of dissolved gases in blood at the knee region is small compared to the amount dissolved in the water and fat, due to the relatively lesser vascularity.

The second component of the inert krypton uptake has a half saturation time T_2 between 10 and 40-50 minutes in the hand and between 35 and 72 minutes in the knee. The amplitude of this com-

ponent in a group of young persons varies between 50 and 66% of the total for the hand and 17 and 42% in the knee region. If there were no third component, to all practical purposes complete saturation of the hand would be reached in about 90 minutes and 150 minutes in the knee.

The third component for the hand has 100 to 320 minutes half time, with the amplitude between 35 and 55%. T_3 varies between 190 and 870 minutes in the knee with an amplitude variation of 61 to 90%.

Table V shows a set of hand data taken on different subjects and Table VI shows the constants of the knee region taken on seven subjects. In the same tables the susceptibility to decompression sickness is shown, as determined in repeated ascents in the decompression chamber by standard exercise methods. Though gas ex-

TABLE VI
Uptake of krypton in the knee region

Name	A ₁ , T ₁ : Small					Bends susceptibility
	Age	First component		Second component		
		A ₂	T ₂	A ₃	T ₃	
			<i>minutes</i>		<i>minutes</i>	
E. F.	21	.41	60	.59	870	s s i r i s i
G. B.	20	.41	54	.59	230	
W. C.	21	.11	36	.89	440	
M. Ch.	21	.39	48	.61	190	
G. J.	19	.29	67	.71	270	
S. C.	21	.17	72	.71	220	
C. T.	25	.43	50	.57	600	
Average	21	.32	55	.64	400	

change has a bearing on the development of decompression sickness, there is no obvious correlation in the data presented. It has been shown with other methods that total resting nitrogen exchange has correlation to bends, but also that there are other important factors to consider (*e.g.*, state of exercise).

The gas exchange curves presented in this paper are reproducible to a certain extent. This conclusion is based on repetitions of the experiment in four subjects. It would appear that under identical experimental conditions and with less than a month interval between consecutive measurements the individual variations of the amplitudes and time constants are less than the variations in a group of the same age. It is essential, however, to note that a change in physiological condition may cause a considerable change in the shape of the gas exchange curve, especially that of the hand.

A number of experimental conditions were provided to study such changes; these proved valu-

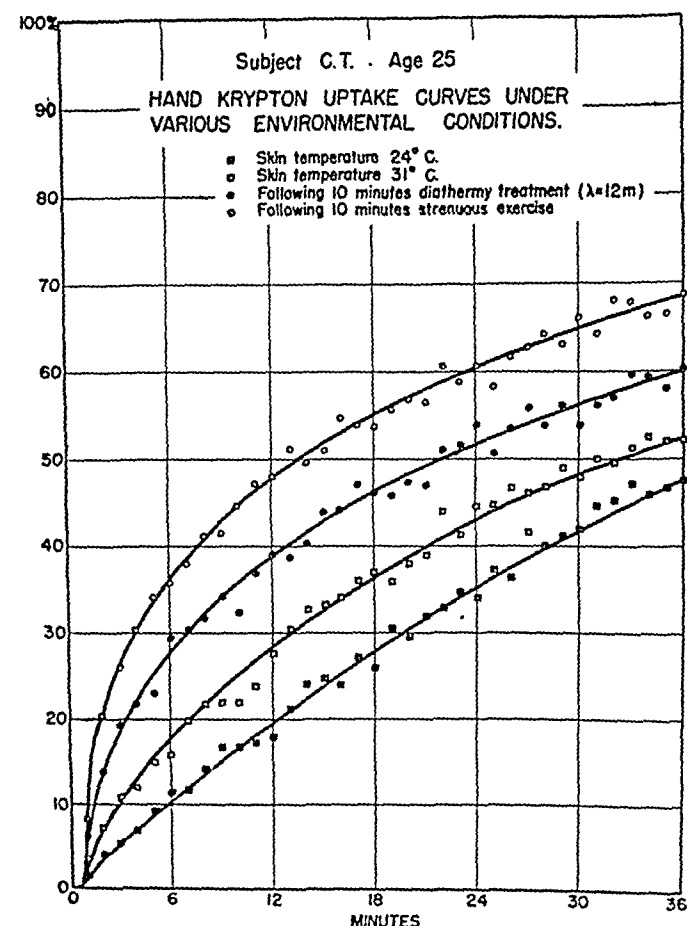


FIG. 6. VARIATIONS IN THE HAND UPTAKE CURVE OF A SUBJECT UNDER VARIOUS ENVIRONMENTAL CONDITIONS

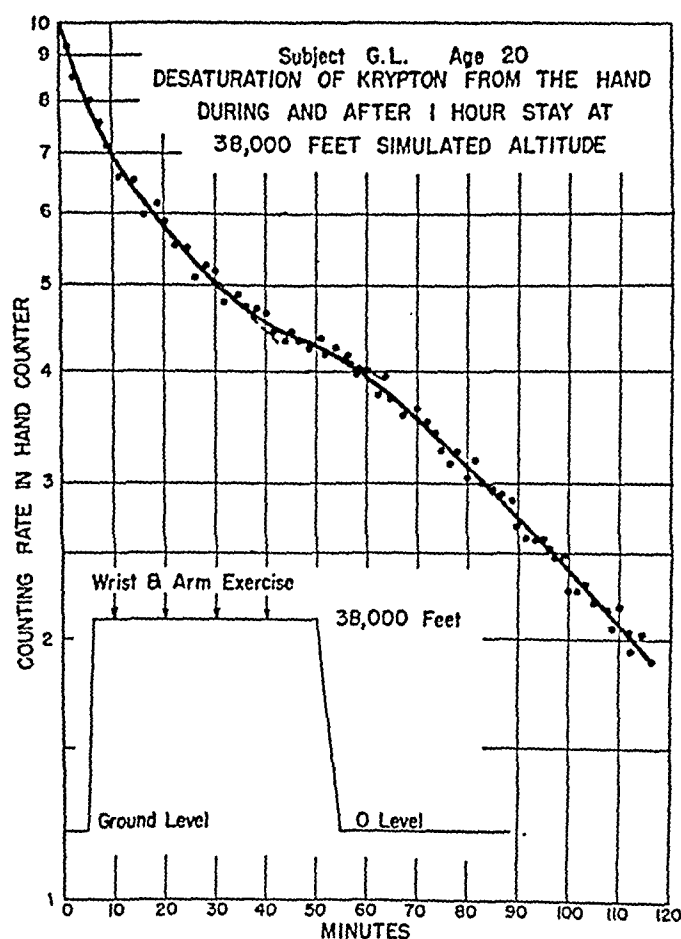


FIG. 7. DEMONSTRATION OF RETARDATION IN THE KRYPTON ELIMINATION OF THE HAND DURING A HIGH ALTITUDE FLIGHT

The rate of elimination returned to normal after return to ground level. Note inflexion in the curve.

able in the interpretation of data. The uptake and desaturation curve of radiokrypton was influenced easiest in the hand. On subjects with cold hands usually a characteristically low gas exchange rate was obtained. This phenomenon appears to be due to a vasoconstriction of the surface blood vessels of the hand. In terms of the exponential components of the uptake curve: A_1 decreased by a factor of three or four (see Figure 6). Diathermy heating of the hand for 10 minutes with a 12 meter wavelength machine increased A_1 above normal, as shown in Figure 6, and it appeared to decrease T_2 . Heavy exercise just prior to the test (10 minutes of fast bicycle riding) increased A_1 to about twofold normal. Administration of adrenaline (0.5 cc. of 1/1000 adrenaline) had the same effect as cold skin temperature. We may thus conclude that rapidity of krypton exchange reflected the state of the vascular bed of the hand. No extensive tests were carried out for the knee

region; it was apparent, however, that the changes were considerably less in magnitude.

The rate of radioactive krypton and argon uptake was studied in a large group of young subjects in short, half-hour tests. An index was derived from these data and correlated with the incidence and severity of decompression sickness. It was found that after exposure to low atmospheric pressure, when the subjects were totally at rest in the decompression chamber, the coefficient of correlation was very high: those with low gas exchange rates got the bends, in contrast to the correlation with the incidence of decompression sickness after exercise (*e.g.*, Tables V and VI) when it was low. Since the blood flow or the carrying capacity of blood is an important factor in the rapidity of the exchange, we attempted to see whether in some way this carrying capacity could be increased. Since nitrogen and krypton are much more soluble in fats and oils than in water, it was thought that lipemia occurring some two hours after ingestion of a fatty meal might help to increase the rapidity of gas exchange, and thus relieve the danger from decompression sickness. Three subjects were given a test of three hours duration two hours after ingestion of a heavy fatty meal.⁵ The rapidity of gas exchange of the hand was not altered in these experiments.

In view of the correlation found between skin temperature of the hand and subsequent development of bends pain we attempted to detect a change in the rate of krypton exchange of the hand during decompression chamber tests. Five subjects were taken to 35,000 feet equivalent altitude for an hour to study the rapidity of krypton desaturation. All these subjects had breathed pure oxygen. Three of the subjects were free from bends pain: their desaturation curve remained normal. One subject, who on many previous occasions invariably developed bends pain in the right shoulder, showed definite slowing down of the rate of hand gas exchange in the decompression chamber, though during his 60 minute stay at high altitude he did not have any pain. The fifth subject, whose desaturation curve is plotted in Figure 7, had incapacitating bends in his left knee, right shoulder, elbow and wrist during the time his gas exchange was measured. There was again a definite slowing

of his desaturation curve in the decompression chamber. After reaching normal atmospheric pressure, the gas rate of krypton exchange curve rapidly reached normal. The changes observed fitted quite well with the general conclusions regarding the state of the circulation in decompression sickness in a different set of experiments (25), but they were not early enough or pronounced enough to be suitable for a preselection test.

It was of some interest to compare the rate of gas exchange observed on the hand of the same subject using different gases: radioactive nitrogen, argon, krypton and xenon. While we are in possession of some data more work needs to be done. The rate of gas exchange as far as components T_1 and T_2 were concerned seemed to be proportional to the solubility of each gas in water, in agreement with Jones' findings for total body uptake. T_3 , however, in the case of xenon appeared to be somewhat larger than the value predicted from solubility considerations alone.

The inert gases dissolved in the body fluids may exchange with the external atmosphere through tissues other than the lungs. Behnke and his collaborators have shown that a small fraction of total helium exchange of the body occurs through the pores of the skin (13). The mucous membranes of the intestines, especially those of the duodenum, play an important role in the secretion of a number of substances. It seemed to be of some interest to test the distribution of gases through these membranes. The gas was introduced to the duodenum by a duodenal tube. A small fraction of the gas from the duodenum rapidly got into the circulation and appeared in the extremities as well as in the exhaled air. A gamma ray counter held in one of the subject's hands recorded the rate of rise of radioactivity in the hand, and the concentration in the exhaled air was indicated by another Geiger counter tube within the spirometer. The three curves obtained on three subjects indicate that in about 15 minutes the concentration of radioactive gas in the hand reached peak value. The curves obtained show wide variation when compared with each other, however, and it is not certain whether they indicate the rapidity of uptake through the duodenal wall or whether they also depend on the nature of the aggregation of gas bubbles within the intestinal space itself. Similar experiments were performed

⁵ This consisted of one milkshake, $\frac{1}{4}$ lb. of butter and one pint of cream.

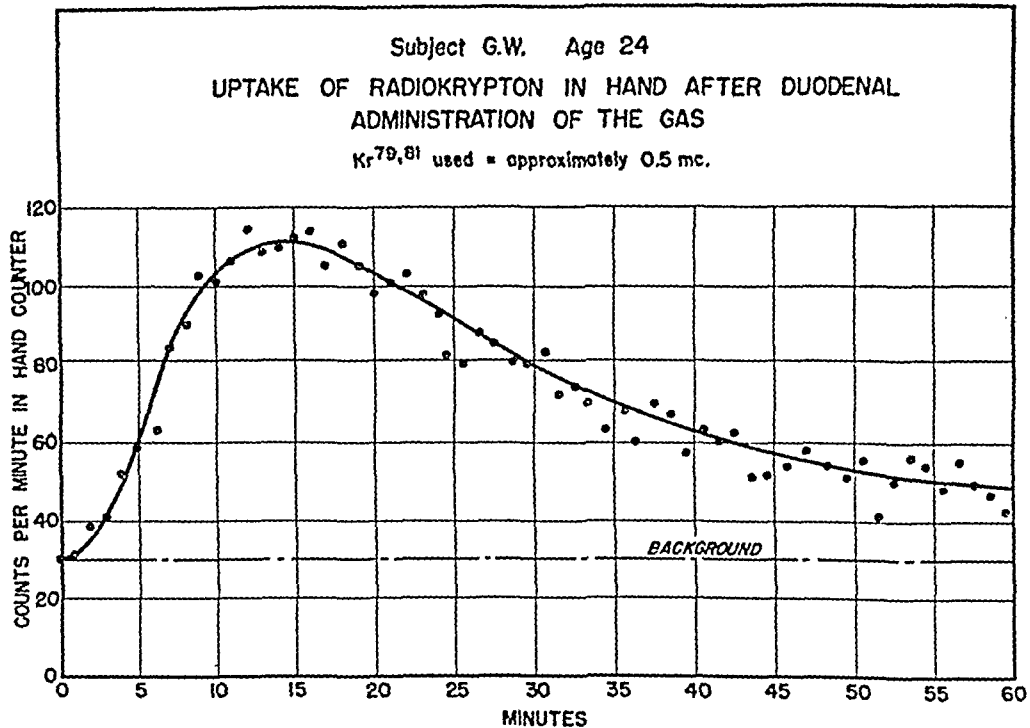


FIG. 8. UPTAKE OF RADIOKRYPTON IN HAND AFTER DUODENAL ADMINISTRATION OF ABOUT 0.5 MC. KRYPTON

with the gas administered to the stomach and to the large intestines. In these latter cases the uptake was not measurable within the first 30 minutes. The rapid exchange of gases from the intestinal tract to the circulation gives good support for recent methods of treatment for intestinal distention. If such intestinal gas consists mainly of nitrogen, then the excess gas will be absorbed by the circulation more rapidly if the patients are given pure oxygen. After a number of minutes of oxygen breathing, the dissolved nitrogen in the body becomes unsaturated and the nitrogen uptake from the intestines becomes accelerated. Other radioactive isotopes may also be used in this way to study the rate of exchange through the intestinal wall in normal and pathologic conditions.

SUMMARY

The rate of change of radioactive krypton concentration in the extremities of young male subjects has been studied. If the subjects breathed a constant concentration of radiokrypton, mixed with oxygen, the uptake and desaturation curves could be satisfactorily expressed as the sum of not more than three superimposed components, changing as the simple exponential function of time.

Exercise or heating of the hand prior to the gas exchange resulted in a generally faster ex-

change. Vasoconstriction or adrenaline caused slower exchange. A fatty meal eaten two hours before the krypton exchange had no effect on the rapidity of the test.

The gas exchange of some subjects slowed down while at 35,000 feet simulated altitude, breathing oxygen. Radioactive krypton, administered via stomach tube appeared rapidly in the circulation of the extremities and in the exhaled air of the lungs.

The techniques and results reported suggest that these radioactive gases have applications in the study of the circulation to the extremities in the living patient and in numerous problems of gas exchange in normal and pathologic states.

ACKNOWLEDGMENTS

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THE HEMODYNAMIC EFFECTS OF HYPOTENSIVE DRUGS IN MAN.

II. DIHYDROERGOCORNINE¹

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Dale in 1906 recognized the sympatholytic activity of crude extracts of ergot as well as their property of direct stimulation of smooth muscle, including that of the blood vessels (1). Despite their sympatholytic effects their direct vasoconstrictor action prevented significant lowering of arterial pressure.

Later chemical purification of the crude drug resulted in the isolation of five alkaloids: ergotamine (2), ergobasine (3), ergokryptine, ergocornine and ergocristine (4). The basic chemical structure of these compounds was clarified by Jacobs and Craig (5) who demonstrated the presence of lysergic acid in all of the alkaloids (6). By hydrogenating the reducible double bond of the lysergic acid component of four of these alkaloids, Stoll and Hofmann (7) in 1943 produced a new series of compounds, dihydroergotamine, dihydroergocristine, dihydroergocornine (DHO-180)⁵ and dihydroergokryptine (DHK-135).⁵ Pharmacological studies in animals (8) indicated that these modified compounds have enhanced sympatholytic and adrenolytic properties but little or no direct constrictor action on smooth muscle. In addition, they are hypotensive, this effect being mediated at least in part via the central nervous system (9). In man DHO and DHK in non-toxic doses have sympatholytic and hypotensive prop-

erties (10-12). Because of their possible value in the treatment of essential hypertension and peripheral vascular disease a further clarification of their hemodynamic effects in man was undertaken.

MATERIALS AND METHODS

The subjects were patients, mostly hypertensive, admitted to the wards of the Massachusetts Memorial Hospitals. The methods used in this investigation were essentially the same as those described in a previous communication (13). DHO was administered by intravenous injection in a solution of sterile water containing 0.5 mg. of active substance per cc. Unless otherwise indicated arterial pressure was measured continuously with a Hamilton manometer (13).

RESULTS

I. Sympatholytic Effects

a. Sympathetic Vasopressor Responses

Certain vasopressor responses usually abolished or diminished after surgical sympathectomy (14)

TABLE I
Effect of DHO on the responses to the Valsalva maneuver

Patient	Control			After dihydroergocornine		
	Arterial pressure		Over-shoot*	Arterial pressure		Over-shoot*
	Basal	After Valsalva		Basal	After Valsalva	
	mm. Hg	mm. Hg	per cent	mm. Hg	mm. Hg	per cent
K. M.	230/130	256/140	10	184/88	180/90	0
F. A.	310/150	330/170	9	238/114	216/108	0
J. D.†	246/112	248/108	0	240/110	230/110	0
J. S.	200/116	260/154	31	188/100	188/98	0
M. T.	180/85	192/88	5	160/74	150/72	0
F. T.†	220/120	220/132	4	208/105	206/100	0
C. H.	186/100	264/152	45	172/98	190/110	11
P. P.	280/150	360/190	28	208/130	200/120	0
P. R.	200/128	244/140	17	150/100	130/80	0
M. I.	192/90	238/110	23	202/92	202/92	0
F. S.	165/100	218/125	39	142/84	120/80	0

* Calculated from the "mean" (one-half the sum of the systolic and diastolic) pressure.

† Following bilateral lumbodorsal splanchnicectomy two years (J. D.) and two weeks (F. T.) respectively.

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⁵ Hereinafter referred to as DHO and DHK respectively.

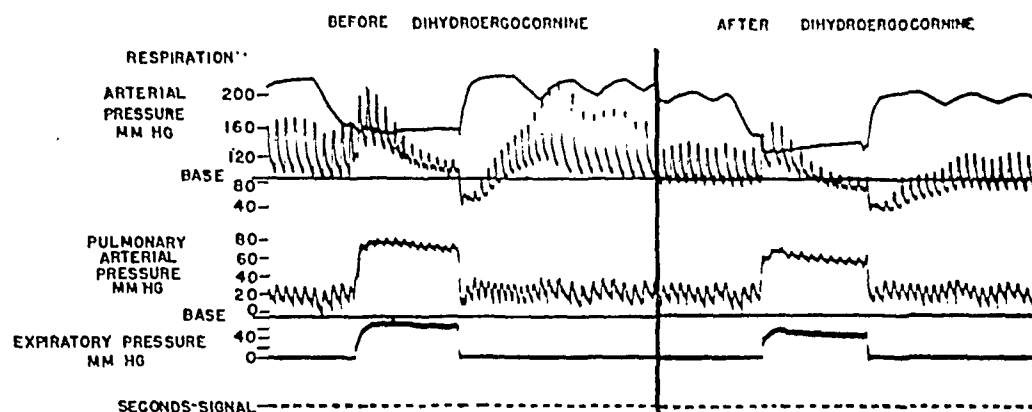


FIG. 1. OPTICAL RECORDS OF THE SYSTEMIC (FEMORAL) AND PULMONARY ARTERIAL PRESSURE DURING THE VALSALVA MANEUVER BEFORE AND AFTER THE INTRAVENOUS ADMINISTRATION OF 0.4 MG. OF DIHYDRO-ERGOCORNINE IN PATIENT F. S.

In the control period there was a marked overshoot of the systemic, but not the pulmonary arterial pressure following the release of the expiratory effort. After dihydroergocornine the overshoot of systemic pressure was completely abolished. In the respiratory tracing inspiration is down.

were completely blocked or definitely inhibited after DHO. These included:

The Valsalva Overshoot

After the intravenous administration of DHO there was complete abolition of the pressor response to the Valsalva maneuver in ten, and marked inhibition in one of 11 patients tested (Table I, Figure 1). The suppression of this vasopressor response developed gradually so that complete abolition did not occur until ten to 20 minutes after the intravenous injection of the drug.

Maintenance of Arterial Pressure During Orthostasis and the Overshoot on Tiltback

After DHO three of four patients with uncomplicated essential hypertension manifested varying degrees of orthostatic hypotension (Table II) while the fourth developed collapse two and one-half minutes after being tilted to an angle of 75°. Two other hypertensive patients who had undergone lumbodorsal splanchnicectomy (J. D. and F. T., Table II) and one normotensive patient with hyperthyroidism (V. C.) had greater postural hypotension after than before the drug. In all

TABLE II

The effect of DHO on the responses to the upright posture and quick tiltback

Patient	Control				After DHO			
	Basal arterial pressure	After four minutes in upright position		Tiltback overshoot mean arterial pressure	Basal arterial pressure	After four minutes in upright position		Tiltback overshoot mean arterial pressure
		Arterial pressure	Increase in heart rate			Arterial pressure	Increase in heart rate	
	mm. Hg	mm. Hg	per cent	per cent	mm. Hg	mm. Hg	per cent	per cent
V. C.*	130/68	126/88	18	24	138/68	119/55	11	0
J. D.†	240/110	208/112	8	1	210/100	162/100	6	0
M. T.	180/85	185/84	5	5	155/78	106/82	0	0
F. T.†	220/122	175/115	44	0	210/107	144/80	28	0
E. M.	203/108	194/110	12	13	175/100	107/54	Bradycardia (collapse)	0
						at two and one-half minutes		
M. I.	190/88	204/94	2	14	192/90	170/92	6	0

* Hyperthyroidism—normotensive.

† Post-sympathectomy.

TABLE III

Effect of DHO on the responses to the cold pressor test

Patient	Control			After dihydroergocornine		
	Arterial pressure			Arterial pressure		
	Basal	Peak response to ice	Increase*	Basal	Peak response to ice	Increase*
	mm. Hg	mm. Hg	per cent	mm. Hg	mm. Hg	per cent
K. M.	218/140	240/156	10	160/82	170/90	7
J. D.†	230/106	283/126	22	234/108	230/115	1
M. T.	160/80	195/90	19	150/76	168/80	10
J. S.	186/96	214/117	18	178/91	188/95	5
C. H.	170/90	194/104	15	150/82	160/87	7
M. M.	93/74	132/94	35	101/79	120/94	19
I. F.	113/81	124/94	12	105/79	112/90	9
C. M.	170/109	202/122	16	155/99	166/116	10
N. McK.	199/129	228/142	9	195/127	206/138	7

*Calculated from the "mean" (one-half the sum of systolic and diastolic) pressure.

†Two years following bilateral lumbodorsal splanchnicectomy.

cases the vasopressor overshoot which normally occurs on tilting quickly from the erect to the supine position was completely abolished by the drug.

The Cold Pressor Response

This response, which is inhibited after dibenamine (15), tetraethylammonium chloride (16), continuous caudal (17) and high spinal anesthesia (18), was partially inhibited in all nine patients tested after as compared with before the administration of DHO (Table III).

b. Digital Thermometric and Plethysmographic Responses

Of six patients examined in a room maintained at 72° F only one exhibited a significant rise in the temperature of the fingers and toes following the drug. On decreasing the room temperature to 68° F the temperature of the digits in this subject fell precipitously to below pre-drug levels. By contrast in three cases examined at a room temperature of 78° F after a control period of more than one hour the intravenous injection of DHO was followed by marked elevations of skin temperature in the digits. Thus, vasodilatation as indicated by rises in skin temperature following DHO appeared to be weak and to depend partly on the temperature of the environment.

Digit plethysmograms were recorded in four patients before and after DHO 180. The pulse volume in both fingers and toes increased after the drug in all four cases, and in three increased markedly. Re-examination of one of these patients seven hours after the drug had been administered revealed a persistence of the marked increase in pulse volume (Figure 2). Reflex vasoconstrictions to "noxious" stimuli (ice, deep breath) were abolished in one case, inhibited in one (Figure 2), and unaffected in one case. In the remaining subject reflex vasoconstrictions after ice, but not after deep breath, were abolished. The normal phasic variations in digital volume were not significantly inhibited in any one of these subjects.

II. Blood Flow Through Various Areas

Blood Flow Through the Forearm and Leg

The changes in blood flow in the limbs with the circulation to the hand and foot excluded (13) were determined before and after the intravenous administration of DHO in 11 hypertensive patients and three normotensive subjects. In the six patients who exhibited the greatest reduction in arterial pressure (Table IV), the mean decrease in arterial pressure was 10.9 per cent while the mean increase in blood flow was 21.3 per cent in the forearm and 10.8 per cent in the leg. Thus, peripheral resistance usually was definitely decreased in this group.

However, the average changes in the hypertensive group as a whole were not as marked. The mean blood flow in the forearm increased slightly (8.3 per cent), from 3.44 cc. per 100 cc. limb volume (range 1.68 to 6.51 cc.) to 3.66 cc. (range 1.43 to 7.80 cc. [Table V]). In the leg the mean blood flow also increased 5.4 per cent, from 3.31 per 100 cc. limb volume (range 1.94 to 5.61) to 3.44 cc. (range 1.66 to 6.20 cc.). The mean arterial pressure exhibited an average decrease of 8.7 per cent (range 1.3 to 19.4 per cent).

Although in most instances peripheral resistance decreased there were certain exceptions. In some cases, the blood flow decreased as the arterial pressure fell. Frequently, the maximum increase in blood flow in the extremities occurred before or after the arterial pressure reached its minimum, and blood flow changes in the forearm and leg often did not occur synchronously or even in the

TABLE IV

Detailed data on effects of dihydroergocornine on the arterial pressure and the blood flow in forearm and leg of the patients who exhibited significant reductions in arterial pressure

Case no.	Patient and diagnosis	Sex	Age	Dose	Control			After DHO-180						
					Arterial pressure	Blood flow cc./100 cc. limb volume		Time after drug	Arterial pressure	Maximum per cent change in mean arterial pressure	Blood flow cc./100 cc. limb volume		Per cent change in blood flow	
						Arm	Leg				Arm	Leg	Arm	Leg
1	L. K. Essential hypertension	F	37	mg. 0.3	mm. Hg 176/150 187/154*	2.03	—	7	167/132	—	3.27	—	— 1.2	—
						3.31*	—	35	163/112	—19.4	3.08	—	— 7.0	—
						—	—	90	166/119	—	3.67	—	+ 10.9	—
2	A. D'I. Essential hypertension malignant phase	M	58	0.4	239/137 238/129 239/129*	4.20	4.18	8	211/118	—	5.32	4.97	+ 11.3	+ 7.8
						4.62	4.58	11	213/115	—10.9	5.47	5.11	+ 14.4	+10.9
						4.78*	4.61*	—	—	—	—	—	—	—
3	C. M. Essential hypertension	F	55	0.25	175/110 169/102 170/109*	1.89	2.50	6	163/99	—	1.51	2.20	— 10.1	—10.6
						1.82	2.46	17	157/101	—	1.43	2.22	— 14.9	— 9.8
						—	—	30	155/95	—10.4	1.52	2.18	— 9.5	—11.4
				0.25 at 34 minutes	—	1.68*	2.46*	53	155/98	—	2.02	2.76	+ 20.3	+12.2
								63	152/96	—11.1	1.97	2.94	+ 17.3	+19.5
								74	155/99	—	1.67	2.52	— 0.6	+ 2.4
4	L. LeL. Essential hypertension	M	48	0.3	160/94 147/96*	2.39	2.41	7	141/90	—	2.39	1.88	+ 6.7	—17.5
						—	—	12	139/88	—	2.38	1.66	+ 6.3	—27.2
						2.24*	2.28*	26	135/87	— 8.6	2.12	1.70	— 5.4	—25.4
						—	—	44	141/91	—	2.28	1.74	+ 1.8	—23.7
5	L. LeC. Essential hypertension	M	48	0.3	243/129 250/129 252/135*	2.53	2.76	7	227/128	— 8.3	2.86	3.35	+ 35.6	+39.0
						—	—	12	238/133	—	3.58	3.96	+ 69.7	+64.3
						2.03	2.27	29	228/127	—	3.86	3.85	+ 83.0	+59.8
				0.15 at 32 minutes	—	2.11*	2.41*	39	231/127	— 7.5	4.38	4.38	+107.7	+81.8
								44	229/129	— 7.5	4.67	4.48	+121.2	+85.9
								60	235/135	—	5.01	4.68	+138.0	+94.2
6	R. I. Essential hypertension	F	39	0.3	168/105 169/101*	2.33	1.86	5	175/105	—	1.86	1.89	— 15.8	— 2.6
						—	—	25	153/97	— 7.4	2.19	1.90	— 0.9	— 2.1
						2.31	1.99	47	153/97	— 7.4	2.55	2.16	+ 15.4	+11.3
						2.21*	1.94*	62	155/97	—	3.09	2.28	+ 39.8	+17.5

* Last control determinations. Post-drug per cent changes in mean arterial pressure and blood flow are calculated from these data. Values for maximum per cent change in mean arterial pressure correspond with other post-drug data on same line.

same direction. Similar inconstant changes in blood flow were observed in three normotensive subjects (Cases 12-14, Table V).

Estimated Hepatic-Portal Blood Flow

The effect of DHO on the estimated rate of hepatic-portal blood flow was studied in four hypertensive patients (Table VI), all of whom reacted with substantial reductions in arterial pressure. In the first patient (F. A.) blood flow was reduced in each of the first three measurements after DHO, but in the fourth had returned to the control range despite a continued moderate reduction of pressure. The second patient (J. C.,

Table VI, Figure 3), five minutes after receiving DHO, had a blood flow rate still within control range in the presence of a considerable reduction in arterial pressure, but in all five subsequent observations during the ensuing 88 minutes had a proportionately greater reduction in flow than in pressure. The third patient (C. B.) resembled the first, while the fourth (C. R.) was similar to the second, except that at 24 and 36 minutes after DHO was given he developed collapse with a blood pressure of 86/50 mm. Hg. There was a spontaneous recovery and the pressure became stabilized at approximately 104/60 mm. Hg. When the effects of DHO had been observed for

more than an hour in this patient, an intramuscular injection of ephedrine sulfate (33 mg.) was given and was followed by an increase in both hepatic-portal blood flow and arterial pressure (Table VI).

All four experiments demonstrated striking re-

ductions in hepatic-portal blood flow following injection of DHO, the maximal fall from the average control level being 37, 73, 61, and 58 per cent, respectively. These were greater than the corresponding reductions in arterial pressure of 23, 20, 33,

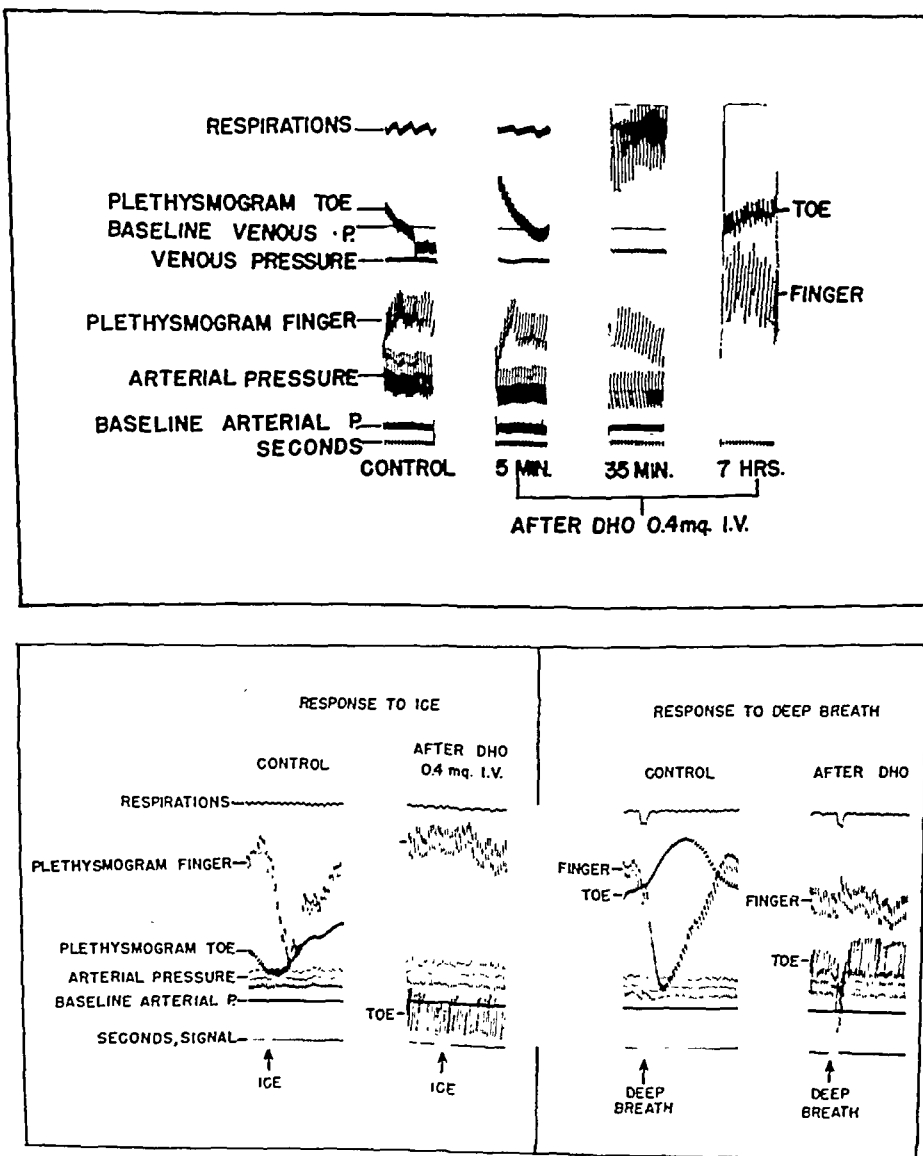


FIG. 2

Optical records, above, of the changes in the antecubital venous pressure, brachial arterial pressure and finger and toe plethysmograms following the intravenous administration of 0.4 mg of dihydroergocornine in patient A. A. Following the drug a marked increase in the pulse volume of both finger and toe occurred which was still evident after seven hours. The antecubital venous pressure rose and exhibited an increase in arterial pulsations.

The lower tracing also illustrates the increase in pulse volume of the finger and toe plethysmograms and, in addition, shows the inhibition of the reflex responses to a deep breath and the application of ice to the forehead after 0.4 mg. of dihydroergocornine in patient E. K.

and 29 per cent, respectively. Vascular resistance appeared to be unchanged or increased in the presence of a reduced level of arterial pressure in 16 of 19 observations on the effects of this drug, suggesting that "splanchnic" arteriolar dilatation was not an important factor in the hypotensive response.

Renal Blood Flow

Of the five hypertensive patients examined two exhibited marked reductions in arterial pressure following DHO (F. A. and K. M., Table VII, Figure 4) and three had moderate reductions. In patient F. A. the arterial pressure fell suddenly from 300/150 to 167/90 over a period of three and one-half minutes after the drug. This sudden hypotensive response was accompanied by a marked

fall in renal plasma flow from 300 to 90 cc. per minute. However, the renal plasma flow returned to control values during the next ten minutes despite only partial return of the arterial pressure. The changes in renal plasma flow followed a similar pattern in patient K. M., while in the remaining three patients, in whom arterial pressure changes were not marked, the renal plasma flow decreased slightly, remaining somewhat depressed in two and increasing in the other after 25 minutes to approximately control levels. Thus, in all five cases renal plasma flow decreased initially; it remained slightly depressed in two and returned to approximately control values in three.

It is of interest that in subject F. A. both renal and hepatic-portal blood flow rates were estimated

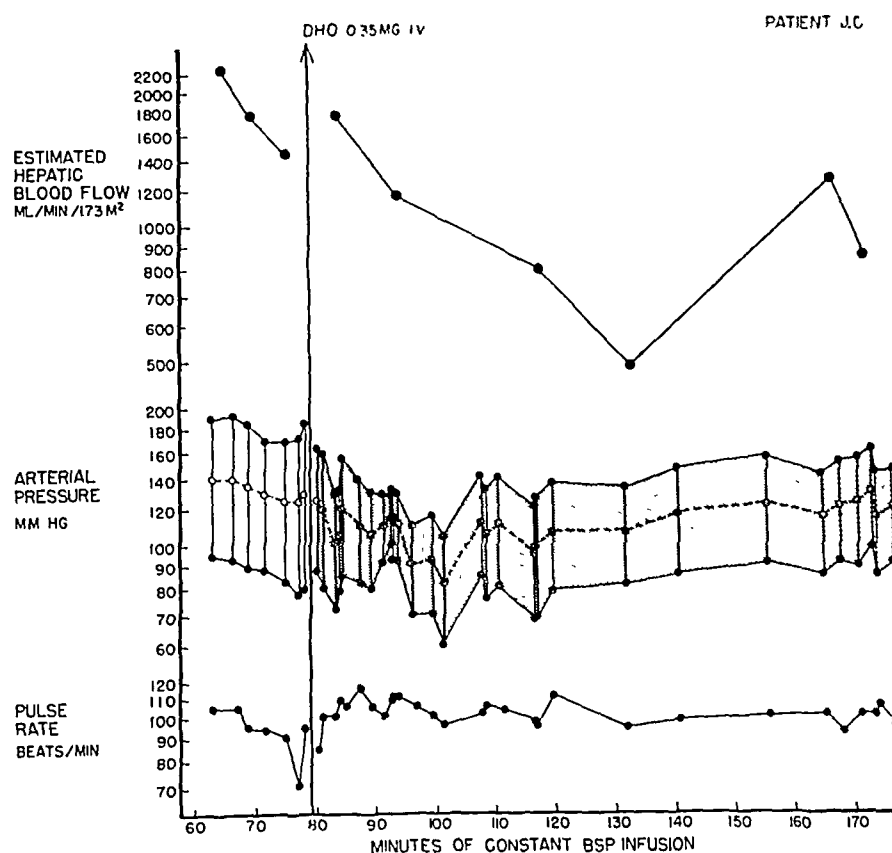


FIG. 3. CHART OF THE CHANGES IN ESTIMATED HEPATIC-PORTAL BLOOD FLOW, ARTERIAL PRESSURE AND HEART RATE (HAMILTON) BEFORE AND AFTER THE INTRAVENOUS ADMINISTRATION OF 0.35 MG. OF DHO IN PATIENT J. C.

Five minutes after DHO during a sudden reduction in arterial pressure the hepatic-portal flow was maintained indicating a decrease in peripheral resistance. Following this initial period, however, the hepatic blood flow fell, gradually returning toward control values 85 minutes after the drug was administered. Calculated "crude peripheral resistance" remained increased following the initial period.

Summary of effects of dihydroergocalciferol on the arterial pressure and the blood flow in the arm and leg

Case no.	Patient and diagnosis	Sex	Age	Dose	Control				After DHO-180					
					Arterial blood* pressure	Blood flow cc./100 (limb)		Arterio-venous oxygen difference	Per cent change in corresponding blood flow		Maximum per cent change in blood flow cc./100 cc. limb volume		Per cent change in corresponding mean arterial pressure	
						Arm	Leg		Arm	Leg	Arm	Leg	Arm	Leg
1	L. K. Essential hypertension	F	37	mg. 0.3	mm. Hg. 187/154	3.31	—	-19.4	- 7.0	—	+ 10.9	—	-16.4	—
2	A. D'I. Essential hypertension malignant phase	M	58	0.4	239/129	4.78	4.61	-10.9	+ 14.4	+10.9	+ 14.4	+10.9	-10.9	-10.9
3	C. M. Essential hypertension	F	55	0.25 0.25 at 34 minutes	170/109	1.68	2.46	-10.4 -11.1	- 9.5 + 17.3	-11.4 +19.5	- 14.9 + 20.3	-11.4 +19.5	- 7.5 - 9.3	-10.4 -11.1
4	L. LeL. Essential hypertension	M	48	0.3	147/96	2.24	2.28	- 8.6	- 5.4	-25.4	- 5.4	-27.2	- 8.6	- 6.6
5	L. LeC. Essential hypertension malignant phase	M	48	0.3 0.15 at 32 minutes	252/135	2.11	2.41	- 8.3 - 7.5	+ 35.6 +107.7	+39.0 +81.8	+ 83.0 +138.0	+64.3 +94.2	- 8.0 - 4.4	- 4.1 - 4.4
6	R. I. Essential hypertension	F	39	0.3	169/101	2.21	1.94	- 7.4 - 7.4	- 0.9 + 15.4	- 2.1 +11.3	— + 39.8	— +17.5	— - 6.7	— - 6.7
7	N. McK. Essential hypertension	M	51	0.3	199/129	4.45	4.77	- 5.8	- 5.2	- 2.9	- 11.2	+ 3.2	- 3.3	- 3.3
8	W. F. Essential hypertension	M	46	0.375 0.25 at 29 minutes	192/116	3.85	3.46	- 5.5 - 2.3	- 25.7 - 17.4	-22.3 -11.6	- 25.6 - 17.3	-22.3 -11.5	- 5.5 - 5.5	- 2.3 - 2.3
9	E. S. ? Chronic pyelonephritis	F	35	0.35	193/124	3.57	2.96	- 4.4	- 25.2	+ 7.1	- 25.2	+12.2	- 4.4	- 4.1
10	E. S. Essential hypertension	M	43	0.4	206/135	6.51	5.61	- 2.9	+ 21.3	+ 5.0	+ 21.3	+10.5	- 2.9	+ 1.5
11	E. S. ? Chronic pyelonephritis	M	29	0.25	151/88	3.21	2.56	- 1.3	+ 4.1	- 3.9	+ 4.1	- 3.9	- 1.3	- 1.3
12	J. D. Normotension early syphilis	M	35	0.3	117/71	1.94	2.18	- 5.3	- 6.7	- 2.3	- 6.7	- 2.3	- 5.3	- 5.3
13	M. M. Normotension lumbar cord transection	F	21	0.3	93/74	1.63	1.54	+12.0	- 6.2	+ 1.3	- 11.0	- 9.1	+ 8.4	+ 7.8
14	J. B. Normotensive	F	26	0.3	106/68	2.87	—	- 3.4	+ 6.3	—	+ 6.3	—	- 3.4	—

* Last control determinations. All post-drug per cent changes in mean arterial pressure and blood flow are calculated from these data. In cases 3, 5 and 8, the second line of post-drug values corresponds to the second dose of DHO-180.

TABLE VI
Effects of dihydroergocornine on estimated hepatic-portal blood flow

Patient	Sex	Age	Control period		After intravenous dihydroergocornine				
			Arterial blood pressure	E. H. B. F.	Dose	Time after drug administration	Arterial blood pressure	E. H. B. F.	Change in crude resistance*
			mm. Hg	ml. per min. per 1.73 sq.m.	mg.	minutes	mm. Hg	ml. per min. per 1.73 sq.m.	
F. A.	M	59	307/157	1457	0.40	3	277/140	1319	0
			305/154	1601		12	235/120	965	+
						23	234/115	1202	0
						33	226/116	1501	-
J. C.	M	58	185/95	2240	0.35	5	132/79	1777	-
			180/89	1765		14	132/92	1172	+
			168/83	1445		38	126/69	808	+
						53	132/81	483	+
						87	142/85	1247	+
						93	154/89	844	+
C. B.	F	53	260/158	1162	0.30	5	205/124	806	0
			267/162	953		10	172/111	418	+
						30	164/108	594	+
						42	160/100	471	+
C. R.	M	50	176/100	1163	0.25	3	154/85	860	0
			176/100	910		7	142/85	758	0
			170/101	874		20	118/75	441	+
						35	93/53	766	-
					†	60	108/62	690	0
						—	—	—	—
						72	135/81	726	0
						85	152/88	1070	-

* Change is recorded as positive or negative values above or below the range of resistance values observed during control period. Variation within control range = 0 change. Crude resistance = average arterial pressure ÷ E.H.B.F.
 † Ephedrine sulfate (33 mg.) was injected intramuscularly at 65½ minutes after the injection of dihydroergocornine.

during a single experiment (Tables VI and VII, Figure 4). The marked fall in average renal blood flow for the first ten minute period of urine collection after injection of DHO, which included the time of sudden fall in arterial pressure, has been noted above. Hepatic-portal blood flow apparently fell similarly, but it was not measured at the time of greatest hypotensive response. However, in the second period, when renal blood flow was completely restored, hepatic-portal blood flow was greatly reduced; though it subsequently increased gradually, to regain the control level 32 minutes after administration of the drug.

III. Renal Function

Glomerular filtration rate usually paralleled the changes in renal plasma flow (Table VII, Figure 4). As a consequence there was little change in the filtration fraction. The most consistent change was in the urine flow which uniformly decreased and remained well below control values, even when

the glomerular filtration rate and renal plasma flow had returned to pre-drug levels.

IV. Cardiac Output, Mean Arterial Pressure and Total Peripheral Resistance

Changes in the cardiac output (catheterization method) in systemic arterial pressure (Hamilton method) and pulmonary arterial pressure were determined in two hypertensive and two normotensive subjects before and after the administration of DHO (Table VIII). In the two hypertensive subjects following the injection of 0.30 and 0.40 mg. of DHO intravenously the mean arterial pressure fell 21 and 10 per cent, while the cardiac output increased 6 and 14 per cent, respectively. As a result the total peripheral resistance fell 25 and 20 per cent, respectively.

Whereas the hypertensive patients were studied only in the supine position, the two normotensive subjects were tested in both the supine and semi-erect positions (tilted upright to an angle of

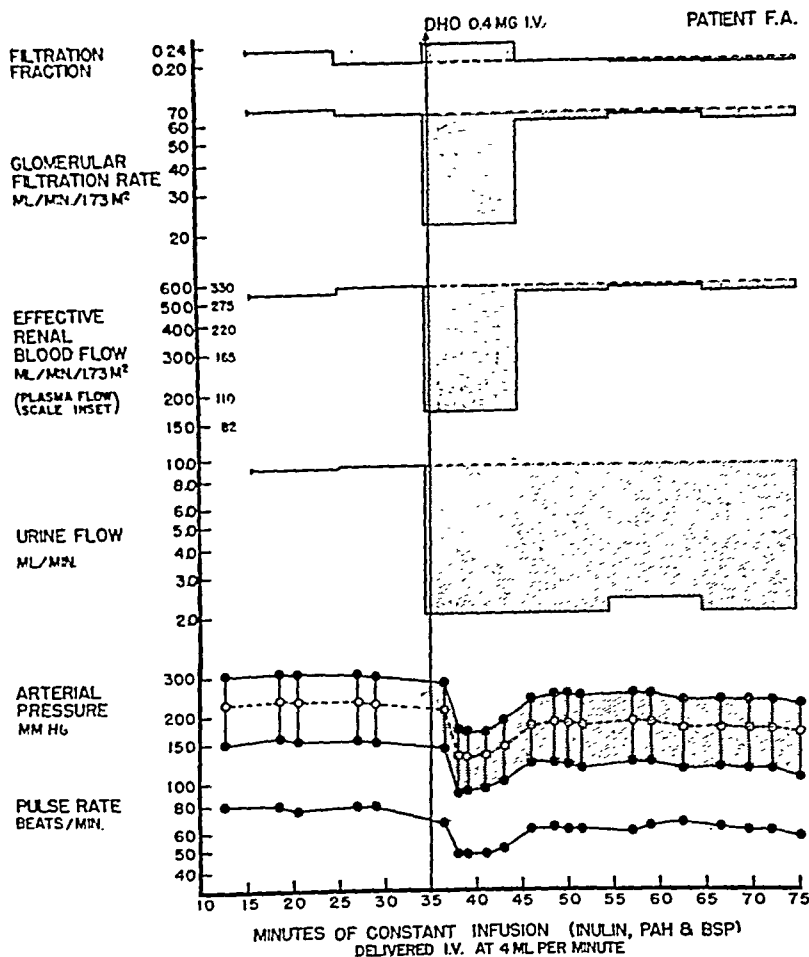


FIG. 4. CHART OF EFFECTIVE RENAL BLOOD FLOW, GLOMERULAR FILTRATION RATE, FILTRATION FRACTION, URINE FLOW, ARTERIAL PRESSURE AND PULSE RATE BEFORE AND AFTER THE INTRAVENOUS INJECTION OF 0.4 MG. OF DHO IN PATIENT F. A.

In the initial period there was a decrease in renal blood flow, glomerular filtration rate and urine flow associated with a reduction in arterial pressure and pulse rate. However, ten minutes after DHO, renal blood flow and glomerular filtration rate returned to control values despite continued significant reduction in urine flow, arterial pressure and pulse rate.

20 to 30°). In the two normotensive subjects, in the supine position, the changes following the drug were qualitatively similar to those observed in the hypertensive subjects. After DHO, the mean arterial pressure decreased 12 and 7 per cent, respectively, the cardiac output increased 11 and 3 per cent, while the total peripheral resistance fell 21 and 10 per cent.

Pulmonary arterial pressure was not significantly changed after DHO in any of the subjects. In the semi-erect position one of the normotensive subjects tolerated the 30° tilt for the five minute

test period without discomfort. Whereas before the drug the mean arterial pressure rose during the tilt, after the drug it fell (postural hypotension). When the table was tilted to 30° the cardiac output fell both before and after the drug, but more so after, and in both instances the total peripheral resistance rose. Thus, in this subject the postural hypotension appeared to be associated with a diminished cardiac output. The second normotensive subject (E. R., Table VIII) was not able to tolerate the 30° tilted position after the drug and developed early signs of collapse in-

TABLE VII
Effect of dihydroergocornine on renal plasma flow, glomerular filtration rate, filtration fraction and urine volume

Patient and diagnosis	Sex	Age	Control						After dihydroergocornine					
			Arterial pressure	Plasma clearance		Filtration fraction	Urine volume	Dose	Time after drug administration	Arterial pressure	Plasma clearance		Filtration fraction	Urine volume
				Inulin	PAH						Inulin	PAH		
F. A. Essential hypertension	M	59	mm. Hg	cc. per min. per 1.73 sq.m.	cc. per min. per 1.73 sq.m.	per cent	cc. per min.	mg.	minutes	mm. Hg	cc. per min. per 1.73 sq.m.	cc. per min. per 1.73 sq.m.	per cent	cc. per min.
			308/156	68.0	299	22.7	8.55	0.4	3.5	167/90	21.8	90	24.3	2.0
			302/153	64.5	320	20.2	9.15		12	235/120	60.0	298	20.1	2.0
									22.5	242/115	62.5	314	19.9	2.3
R. D. Essential hypertension	M	49	192/106	76.4	369	20.7	7.16	0.37	4	188/102	58.8	265	22.2	3.81
			194/106	86.1	344	23.7	6.38		15	182/100		256		3.335
									23.5	176/96	54.2	230	23.6	1.04
									36.5	176/98	86.0	347	24.8	2.19
M. G. Essential hypertension	M	49	220/122	130	510	25.5	9.9	0.25	4	220/118	97.5	518	18.8	7.8
			222/122	143	680	21.0	9.43	0.25 at six minutes	15	212/116	116.5	569	20.5	9.38
									17	200/116	108	418	25.8	3.99
									42	196/120	112.8	488	23.1	2.49
W. W. Essential hypertension	M	20	142/96	95.2	605	16.2	1.045	0.25	1.5	144/96	102.5	492	20.3	0.69
			140/98	91.7	540	17.4	0.875	0.25 at eight minutes	7	126/88	65.0	300	21.1	0.4
									16	138/92	86.1	453	18.5	0.7
									31	136/90	97.2	482	19.7	0.67
K. M. Essential hypertension	F	52	178/88	76.5	383	20.0	1.76	0.30	3	170/88	103.5	392	26.4	2.2
									12	142/80	68.5	270	25.4	0.625
									42	135/80	42.0	212	19.8	0.42
									60	155/82	94.0	367	25.6	1.25

TABLE VIII
Effect of DHO on blood pressure, pulmonary arterial pressure, cardiac output and total peripheral resistance

Patient and diagnosis	Sex	Age	Control						After DHO					
			Mean arterial pressure	Cardiac rate	Pulmonary arterial pressure	Cardiac output	Total peripheral resistance	Dose of DHO	Time after drug	Mean arterial pressure	Cardiac rate	Pulmonary arterial pressure	Cardiac output	Total peripheral resistance
J. H. Essential hypertension	M	40	mm. Hg	per minute	mm. Hg	liters per minute	dynes cm. 5-sec.	mg. I.V.	minutes	mm. Hg	per minute	mm. Hg	liters per minute	dynes cm. 5-sec.
			132	83	31/13	6.65	1590	0.30	9	104	78	33/15	6.28	1320
F. S. Essential hypertension	M	58	132	80	31/6	6.35	1670		23	105	76	28/6	7.53	1120
F. M. Normotensive primary syphilis treated	M	21	130	93	31/8	7.01	1480	0.40	4	124	83	33/14	8.59	1160
			133	95	31/12	6.61	1610		16	114	83	28/13	6.94	1320
E. R. Normotensive primary syphilis treated	M	21	104.8	117	17/5	7.36	1150	0.25	5	98.0	106	17/9	7.60	1030
			108.6	117	26/10	7.08*	1230		15	82.8	111	27/10	6.12*	1070
	M	21	104.3	71	30/11	5.90	1430	0.25	9	90.7	73	29/13	6.55	1105
			100.2	95	38/21	6.13*	1310		27	87.8	88	28/11	9.20†	765

* Patient tilted upright 30°.

† Patient tilted upright 20° (Recovering from collapse which had occurred at 30° upright).

cluding weakness and sweating. He was tilted back to an angle of 20°, a position he could tolerate, and the determinations were then carried out. The mean arterial pressure fell slightly from 91 mm. supine to 88 mm. Hg during the 20° tilt, but the cardiac output increased markedly from 6.55 liters at 0° to 9.2 liters per minute at 20°. Since the patient was recovering from incipient collapse during this period the increase in cardiac output may have been associated with compensatory reactions occurring with the recovery.

V. Other Effects

Heart Rate

A decrease in heart rate following DHO was noted in the present cases as well as in subjects studied previously (10, 11, 19) (Table VIII, Figure 4). In addition, the reduction of arterial pressure in the erect position after DHO, unlike that after lumbodorsal splanchnicectomy or the administration of tetraethylammonium, was not accompanied by tachycardia (Table II).

The bradycardia was only partially abolished by the administration of doses of atropine sulfate which ordinarily produce significant tachycardia in man (0.75 to 1.0 mg. intravenously). In four patients in whom atropine was injected following DHO the acceleration of heart rate varied from 6 to 19 beats per minute. In no instance was the administration of atropine followed by definite tachycardia.

The Response to Venous Congestion of the Extremities

Investigations in this laboratory (20) have disclosed that drugs which cause postural hypotension may cause similar hypotension and collapse in patients who are supine when the venous return from the extremities is compromised by inflating at slightly below diastolic pressure pneumatic cuffs placed proximally about the thighs and one upper arm.

Prior to the drug three hypertensive subjects tolerated such congestion of the extremities for five minutes with no significant alterations in arterial pressure. However, after 0.5 mg. of DHO intravenously two of the three subjects developed hypotension, bradycardia and collapse between four and five minutes after the application of sim-

ilar congesting pressures to the same regions. The significance of these results with respect to the mechanism of the collapse and the effects of other drugs will be reported in another communication (20).

Effect of DHO and DHK on the Response to the Intravenous Administration of Epinephrine

The dihydrogenated alkaloids of ergot are reported to inhibit the pressor response to epinephrine in the anaesthetized experimental animal (8). Similarly in unanaesthetized animals (8) and man (11), DHK may block the hyperglycemic response to epinephrine.

The adrenolytic properties of DHO and DHK on cardiovascular functions were investigated by the use of gradually increasing intravenous doses of epinephrine instantaneously injected. As noted previously in this laboratory (21), the characteristic response to minimal (0.5 to 1 µg.) doses of epinephrine thus administered is mainly depressor and is accompanied by cardiac acceleration and a slight increase in the antecubital venous pressure. After slightly larger doses of epinephrine the depressor response, while still present, is followed by a later pressor phase which increases in magnitude and duration with the dose of epinephrine.

Both DHO and DHK inhibited the depressor phase when single doses of 0.5 to 1.0 µg. of epinephrine were administered intravenously (Table IX). The inhibition was least evident in the response of the arterial pressure, more apparent in the heart rate, and most evident in the response of the venous pressure. However, with increasing doses of epinephrine (2 µg.) inhibition of the depressor phase was not consistent. Further, in the pressor phase of the epinephrine response the rise in arterial pressure was not significantly altered by either DHO or DHK, although the tachycardia and rise in venous pressure were slightly inhibited. Thus, in contrast with the results obtained in experimental animals (8), the dihydrogenated alkaloids of ergot in the doses used appeared to have only slight adrenolytic activity with respect to these cardiovascular functions in man. It is of interest, however, that in a patient with pheochromocytoma both a continued hypertension as well as a marked pressor reaction

TABLE IX

Effect of DHK or DHO on depressor and pressor phases following intravenous administration of small doses of epinephrine

Patient and dose of epinephrine	Control			After DHK or DHO		
	Mean arterial pressure* per cent change	Heart rate per cent change	Venous pressure† per cent change	Mean arterial pressure* per cent change	Heart rate per cent change	Venous pressure per cent change
<i>Depressor phase</i>						
C. P. (1.5 mg. DHK I.V.)						
2.0 µg.	-22	+24		-18	+5	
2.0 µg.	-12	+9		-22	+10	
4.0 µg.	-10	+29		-11	+27	
F. G. (1.5 mg. DHK I.V.)						
0.5 µg.	-11	+16	+5	0	0	0
1.0 µg.	-16	+22	+5	-14	+5	0
2.0 µg.	-23	+30	+12	-22	+22	0
5.0 µg.	-16	+41	+18	-18	+38	+11
8.0 µg.	-14	+29	+16	-17	+36	+10
M. H. (0.3 mg. DHO I.V.)						
0.5 µg.	-14	0		-2	0	
1.0 µg.	-12	+10		-3	+7	
2.0 µg.	-11	+20		-10	+13	
4.0 µg.	-16	+33		-11	+36	
5.0 µg.	-15	+29		-6	+20	
10.0 µg.	-9	+40		-9	+40	
<i>Pressor phase</i>						
C. P. (1.5 mg. DHK I.V.)						
2.0 µg.	+10	+17		+5	0	
2.0 µg.	+4	+5		+3	0	
4.0 µg.	+10	+11		+12	+5	
F. G. (1.5 mg. DHK I.V.)						
0.5 µg.	0	0	0	0	0	0
1.0 µg.	0	0	0	0	0	0
2.0 µg.	0	+7	+6	0	+7	0
5.0 µg.	+5	+14	+12	+7	+3	+5
8.0 µg.	+4	+10	+6	0	0	+5
M. H. (0.3 mg. DHO I.V.)						
2.0 µg.	0	0		+13	0	
4.0 µg.	+6	0		+13	0	
5.0 µg.	+3	+5		+9	0	
10.0 µg.	+12	+5		+17	+5	

* One-half the sum of the systolic plus the diastolic pressure $\left(S + \frac{D}{2}\right)$. Hamilton manometer recordings from the brachial artery.

† Hamilton manometer recordings from the antecubital veins.

to the histamine test were promptly relieved by intravenously injected DHK (22).

Since it has been suggested that the pressor and cardio-accelerator responses to emotional stimuli may be due at least in part to epinephrine discharge (23) it was considered of interest to de-

termine the effect of the emotional stimulus of a problem in mental arithmetic on the arterial pressure before and after DHO. Of three patients tested the pressor response was reduced in two after as compared with before DHO, while the accelerator reaction was reduced in all. These re-

sults must be interpreted with caution, however, since such stimuli tend to lose emotional impact on repetition.

"Vascular Distensibility" in the Extremities

At intervals during the studies of limb blood flow the "vascular distensibility" (increase in volume during graded venous congestion) in the forearm and leg was determined in 14 subjects according to the method of Litter and Wilkins (24). In almost all cases there was a significant decrease in the vascular distensibility, which occurred more consistently than any of the changes in limb blood flow, but did not correlate with either the fluctuations in arterial pressure or the blood flow. The significance of these findings will be discussed elsewhere (24).

Venous pressure in the antecubital vein, recorded in four subjects with a Hamilton manometer, rose 1 to 4 mm. of mercury, after DHO. The marked increase in peripheral venous pressure during the Valsalva maneuver was also considerably greater after as compared with before DHO. In addition, arterial pulsations became more prominent in the antecubital venous recordings after the drug (Figure 2).

DISCUSSION

This investigation supplied additional evidence confirming the conclusions of Bluntschli and Goetz (10) that DHO has sympatholytic effects in man. It disclosed that DHO not only inhibits vasoconstriction in the extremities, but also depresses vasopressor responses mediated over sympathetic pathways.

The bradycardia which frequently follows the injection of the dihydrogenated ergot alkaloids also appeared to be due in part to inhibition of the sympathetic cardio-accelerator mechanism, since after DHO doses of atropine sufficient to inhibit vagal influence on cardiac rate failed to produce significant tachycardia. In contrast to their apparent sympatholytic effects, the dihydrogenated alkaloids in the doses used had only slight adrenolytic properties so far as the immediate cardiovascular reactions to injected epinephrine were concerned. Such evidence suggests that the circulatory changes observed in the present studies in man may have been due to the central action rather

than the peripheral sympathetic-blocking effects of these drugs (8, 9).

The fall in arterial pressure following DHO, except in the semi-erect position, was associated with maintenance of cardiac output, and, hence, with a decrease in total peripheral resistance. The maintenance or slight increase in cardiac output may have been influenced by any one or a combination of the following factors: 1) the maintenance of right cardiac filling pressure as evidenced by the rise in peripheral venous pressure and unchanged pulmonary arterial pressure, 2) the cardiac slowing which permitted more effective diastolic filling, and 3) the diminished peripheral resistance. A similar hemodynamic response was observed after veratrum viride (13), the action of which also involves the vascular centers in the nervous system. Such a response is in marked contrast to that caused by certain peripherally acting vasodilators such as sodium nitrite after which cardiac output falls in association with compensatory tachycardia and a decrease in venous and pulmonary arterial pressure (25).

Although *total* peripheral resistance decreased uniformly after DHO the pattern of blood flow in discrete peripheral areas exhibited marked fluctuations. In the renal area the blood flow decreased early, but later increased to or toward control values. Blood flow in the muscles usually increased and in the splanchnic area usually decreased, but these changes were inconstant. Thus, peripheral resistance in different regions might increase, decrease, or vary from one direction to the other during a reduction in blood pressure. In this regard it is of interest that Hafkenschiel and his co-workers (26) found that cerebral vascular resistance was reduced after DHO in hypertensive, but not in normotensive subjects.

This lack of uniform response may indicate that the hypotensive effects of the drug had called forth counteracting mechanisms; or that the drug itself had multiple effects, including opposing actions on the blood vessels. The constrictor action on arteriolar muscle that is exhibited by the naturally occurring ergot alkaloids is said to be minimal with the dihydrogenated derivatives in animals (8), and in the digits of man (10). However, it is possible that in the muscular and hepatic-portal areas of man the direct vasoconstrictor

effect may become active and at times may be occasionally dominant.

Either hypothesis, 1) activation of compensatory vasoconstrictor mechanisms, or 2) multiple opposing actions of the drug, could explain not only the variable changes in blood flow in the splanchnic bed and muscles, but also the apparent failure of DHO to bring about a hypotensive response in certain patients despite evidence of inhibition of sympathetic reflex vasoconstriction (11). The possibility that the hypotensive effect of the drug may be due to some property other than a sympatholytic action also cannot be excluded.

The only peripheral areas studied which manifested a consistent pattern of vasodilatation were the hands and feet when the subjects were examined in a warm room. A decrease in peripheral resistance was manifested by a rise in skin temperature, an increase in pulse volume, and by the presence of arterial pulsations in the venous pressure recordings taken from the antecubital vein. The rise in antecubital venous pressure after DHO in all probability was secondary to the increased arterial inflow, resulting from the local arteriolar vasodilatation without a correspondingly increased venous outflow. The increased pulsation in the veins could have been due partly to transmission from neighboring arteries across intervening tissues. However, this seems unlikely since 1) the pulse in the larger arteries, such as the brachial and radial, became less, rather than more bounding after the drug, 2) the increased pulsation also was observed in superficial veins remote from larger arteries, and 3) since there was obvious evidence of peripheral arteriolar vasodilatation, particularly in the digits, transmission of the pulse waves through the dilated vascular beds to the veins seems a more likely explanation for their presence in the venous tracings.

There were many points of similarity between DHO and tetraethylammonium. Both drugs inhibited sympathetic vasoconstrictor reflexes (27), raised skin temperature in the digits (28), produced an initial decrease in renal clearances followed by a return to control values (29), and as a rule increased limb blood flows (28, 30). The chief points of difference were that after tetraethylammonium the cardiac output either decreased (31) or remained unchanged (28), the

heart rate increased (32) and the pulmonary arterial pressure decreased (31). The reason for these differences is not clear unless they were related to the different sites of action of the two drugs (8, 33).

It was of interest also to compare the effects of DHO with those of veratrum viride, since the hypotensive action of both drugs is believed to be mediated through the central nervous system (9, 34). The two drugs were similar, in that both could decrease arterial pressure and heart rate without depressing cardiac output, they both produced oliguria, and brought about a decrease in renal blood flow during the early, falling phase of the hypotensive response. They were dissimilar in that following suitable doses of veratrum viride significant hypotension occurred almost without exception, the changes in peripheral resistance were uniform in all the vascular areas studied, and the homeostatic vasoconstrictor reflexes remained intact. Atropine abolished the bradycardia induced by veratrum, but not that which followed DHO, while "vascular distensibility," in the extremities, which increased following veratrum, usually decreased after DHO.

Clinically these studies suggest that sympatholytic agents have certain fundamental limitations in the treatment of essential hypertension. First, as has been observed with other agents that inhibit sympathetic vasoconstrictor reflexes (32, 35, 36), not all patients had a significant reduction in arterial pressure following the administration of DHO. Thus, the therapeutic use of this agent, as of other sympatholytic drugs, would seem to be limited to selected patients. Second, its apparent blockade or inhibition of reflex vasoconstrictor mechanism so interfered with homeostasis that at times vascular collapse seemed imminent or actually occurred. Finally, after it had been given, the changes in peripheral resistance in different vascular beds were so varied and unpredictable as to suggest that certain factors opposing a uniform vasodilatation were active.

SUMMARY AND CONCLUSIONS

Dihydroergocornine (DHO) administered intravenously in man produced the following hemodynamic effects:

1. Vasopressor responses to blood-pressure-lowering procedures (Valsalva maneuver, upright tilting), and to the immersion of a hand in iced water (cold test) were inhibited or abolished. Skin temperature in the digits exhibited significant increases in a warm, but not in a cool environment. Digital pulse volumes increased, and reflex vasoconstrictor responses to "noxious" stimuli usually decreased.

2. Blood flow through the forearm and leg frequently increased as the arterial pressure fell, but it sometimes decreased or remained unchanged.

3. Blood flow through the hepatic-portal circuit also varied in different patients, occasionally remaining at control levels early in the hypotensive response, but usually becoming reduced proportionately more than the arterial pressure. The variability of the responses in blood flow in the limbs and in the hepatic-portal region suggests that the drug may have multiple and opposing effects, or that after it has been given, certain counteracting vasoconstrictor mechanisms may become activated.

4. Renal blood flow decreased initially, but in three of five patients it later returned to control levels. Glomerular filtration rate paralleled renal blood flow. Hence, there was little change in the filtration fraction. Oliguria occurred and persisted even when renal plasma flow and glomerular filtration rate returned to control values. Therefore, the oliguria appeared to be secondary to increased tubular reabsorption of water.

5. The fall in arterial pressure in the supine position was accompanied by no change or a slight increase in cardiac output. Total peripheral resistance, therefore, was reduced. The bradycardia accompanying the hypotensive response was not abolished by atropine.

6. Venous congestion by cuffs high on the extremities could precipitate collapse in supine patients who had received DHO.

7. "Vascular distensibility" in the limbs usually decreased, while venous pressure in the arm rose. Arterial pulsations became more prominent in the venous pressure recordings taken from the ante-cubital vein.

8. The alkaloids, dihydroergocornine and dihydroergokryptine, opposed only slightly the effects of epinephrine on the arterial pressure and heart rate of man.

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THE EFFECT OF A CATION EXCHANGE RESIN ON ELECTROLYTE BALANCE AND ITS USE IN EDEMATOUS STATES

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In ancient Greece, "terra sigillata" or "sealed earth" was reputed to be a successful counter agent for poisons (1). Its fame grew through the centuries commensurately with the refined custom of poisoning one's dinner partner.

Early trials of the effectiveness of "terra sigillata" are reported in two 16th century documents (2). The first, dated 1580, records that of eight dogs who were fed various poisons, including mercury sublimate, the four that received "terra sigillata" survived, while the others died. A 1581 account details another successful experiment in which the "earth" was administered to a condemned criminal immediately following "a dram and a half of Mercurie Sublimate."

"Terra sigillata" was found, in 1914, to consist of oxides of calcium and aluminum with a large proportion of silicates (2). Thompson, the analyst, attributed "its virtues . . . chiefly due to the mystery surrounding its origin and the superstitions connected with its source." In the light of more recent knowledge, however, the action of "terra sigillata" may well be explained on the physico-chemical grounds of an ion exchange.

Ion exchangers are insoluble substances which remove ions from solution by releasing one ion in exchange for another (3-6). Way, in 1850, first observed the phenomenon of ion exchange in soil (7). Subsequently a number of naturally occurring substances were found to have cation exchange properties but were of limited use because of their low exchange capacity and chemical instability (8, 9). Advances over the naturally occurring substances were the development of "zeolites" from sodium aluminate and sodium silicate (10-13), and of synthetic organic exchangers from sulfonated coals (14). In 1934, Adams and Holmes prepared synthetic phenol-formaldehyde resins with cation exchange properties and introduced resins prepared from aromatic amines and formal-

dehyde which were capable of exchanging anions (15).

In 1945 anion exchange resins, which remove such ions as chloride, sulfate, and phosphate from solution, were utilized in the therapy of peptic ulcer (16-18). The next year Dock suggested that cation exchange resins, which remove such ions as sodium, potassium, calcium, and magnesium from solution, be used to take up sodium from ingested food. He showed that the particular resin he used was non-toxic in dogs and rats when fed in massive doses over a two-week period (19).

The present study concerns observations of electrolyte interchange between a cation exchange resin and medium, *in vitro* and *in vivo*. The resin was also evaluated in the therapy of congestive heart failure and cirrhosis of the liver by comparing measures needed to control accumulation of fluid with and without resin. The resin used was Liquonex CRW¹ passed through a 120 mesh screen. The insoluble cation exchange resin is a sulfonated polystyrene capable of neutralizing from 3.9 to 4.5 mEq. of base per gram of resin.

PROCEDURES

In vitro: Solutions of known composition were mixed with the resin and the surrounding fluid analyzed in order to determine the proportions of various ions removed under a variety of circumstances.

In vivo: A constant diet was fed to a subject and the effect of oral administration of resin on his electrolyte balance was observed. The subject, a 59 year old male, had neither history nor sign of cardiac or renal dysfunction although he had a mild systolic hypertension and a right hemiparesis. He was cooperative enough to consume the following three meals daily for 80 days. Breakfast consisted of tomato juice, two eggs, a slice of bread with a pat of butter and a glass of milk. Dinner and supper consisted of two ounces of ham, a potato, one

¹ The authors are indebted to Dr. Sidney Sussman (Liquid Conditioning Corp., Linden, N. J.) for the preparation and supply of this resin. Liquonex CRW is essentially Dowex 50.

slice of bread with a pat of butter, a glass of milk and grapefruit. On analysis the diet was found to contain 112 mEq. of sodium, 87 mEq. of potassium, 47 mEq. of calcium, 118 mEq. of chloride, 44 mM of inorganic phosphorus, and 11.9 grams of nitrogen (75 grams of protein). Water was allowed ad libitum.

After a period of 20 days on the diet an amount of resin was administered each day equivalent to 100 mEq. of base. One-third of the daily dose of resin was ingested in plain gelatin capsules during each meal. After nine days the dose was increased to 150 mEq. Twelve days later the dose was increased to 200 mEq. and then in eight days to 250 mEq. After six days on 250 mEq. the resin was discontinued and the patient was observed for an additional 25 days.

The subject was weighed daily. Twenty-four hour urines were collected under oil using toluene as a preservative. The urinary specific gravity, volume, creatinine, pH and titratable acid, total nitrogen, ammonia, sodium, potassium, calcium, inorganic phosphorus, sulfate and chloride were determined. The feces were collected in three-day periods, dried under an infra-red lamp, weighed and ground to a fine powder in a mortar and analyzed for total nitrogen (less ammonia nitrogen), inorganic phosphorus and chloride. Aliquots of the dried feces were treated with concentrated sulfuric acid, ashed overnight at 800° C and analyzed for sodium, potassium and calcium. At 10-day intervals determinations were made of the plasma sodium, potassium, calcium, phosphorus, carbon dioxide combining power, chlorides, pH, urea nitrogen and alkaline phosphatase.

Urinary specific gravity was determined with a urinometer. Urinary pH was determined under oil and the urine was then titrated to pH 7.4 with 0.02 N sodium hydroxide. Sodium and potassium were determined with an internal standard flame photometer (20). Calcium was measured by dissolving the precipitated oxalate in 2 N sulfuric acid and adding an excess of hexanitratammonium cerate. The cerate was back titrated with ferrous ammonium sulfate using ortho-phenanthroline ferrous sulfate as an indicator. Chloride was determined according to Wilson and Ball (21); phosphorus accord-

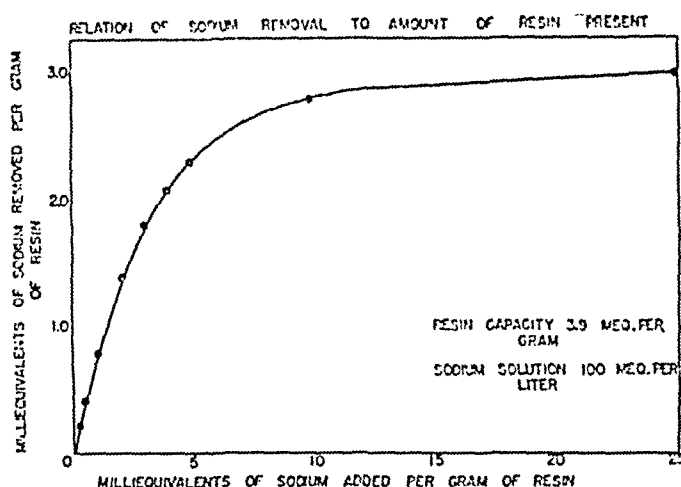


FIG. 1

ing to Fiske and Subbarow (22); ammonia and urea according to Summerson's modification of the Van Slyke and Cullen procedure (23); creatinine by a modification of the Folin method (24). Sulfate was determined by a turbidimetric method using barium chloride (25). Total nitrogen was determined by the micro-Kjeldahl technique. Phosphatase was determined by the King and Armstrong method (26).

OBSERVATIONS

In Vitro

Liquonex CRW like other hydrogen exchange resins removes sodium from solution by forming an insoluble combination of sodium and resin, releasing a free hydrogen ion for each sodium ion fixed to the resin.

As the amount of sodium added per gram of resin was increased, more sodium was removed per gram until the resin was saturated (Figure 1). The volume of water in which the sodium was dissolved had little effect on sodium removal by the

TABLE I

The removal of calcium, potassium and sodium from solution by Liquonex CRW

Solution				mEq. added per gram of resin*			Per cent of added ion removed from solution		
Medium	Ca	K	Na	Ca	K	Na	Ca	K	Na
	mEq./L	mEq./L	mEq./L	mEq.	mEq.	mEq.	per cent	per cent	per cent
Water	26	29	30	0.69	0.79	0.81	100	86	43
0.93N HCl	26	29	31	0.71	0.79	0.83	100	77	19
0.3N NaOH	26	29	31	0.69	0.79	0.84	100	91	58
Water	15	40	55	0.41	1.07	1.47	98	81	44
Water	250	389	552	0.75	1.29	1.84	92	74	29
Milk pH 5.0	13	41	51	0.36	1.10	1.38	96	81	43
Milk pH 6.8	18	41	54	0.49	1.09	1.46	94	82	54
Milk pH 7.5	19	39	57	0.51	1.04	1.53	92	82	59
Water	5	5	140	0.08	0.07	2.05	100	86	64

* Resin capacity 3.9 mEq. of base per gram

resin. Studies of combinations of sodium and resin which would approximate the ratio encountered in the intestine revealed the resin to be incompletely saturated with sodium. Therefore, if the amount of salt is increased in a diet containing a constant amount of resin, a goodly proportion of the increment will be fixed to the resin.

Ions of higher valence or higher molecular weight tend to occupy the resin in preference to ions of lower valence or lower molecular weight (Table I) (27). The resin had a greater affinity for potassium and calcium than for sodium regardless of the medium in which these ions were dissolved. The capacity to remove cation was enhanced in alkaline solution. Sodium, potassium, and calcium in solution in the proportions present in the diet did not affect the preferential removal of potassium and calcium over sodium.

Sodium and potassium chloride were added to milk to approximate the proportions of sodium, potassium, and calcium in the diet. The pH of this solution was 6.8. Ultrafiltration by a Sims ultrafilter revealed 56 per cent of the calcium to be bound to protein. The same solution was also studied after the pH had been adjusted to 5.0 with dilute acid and to 7.5 with dilute alkali. None of these manipulations affected the affinity of the resin for potassium and calcium over sodium. Calcium bound to the protein left the protein to occupy space on the resin.

Using a solution in which the ratio of ions was such as is found in the fluid of the ileum (140 mEq. of sodium, 5 mEq. of potassium, and 5 mEq. of calcium), 64 per cent of added sodium was removed. In this instance, 1.32 mEq. of sodium were removed per gram of resin whereas the maximum amount of sodium removed per gram of resin in the other combinations was 0.8 mEq. per gram. This is a result of the higher ratio of sodium to calcium and potassium.

The action of a cation exchange substance is not limited to inorganic cations alone but basic organic materials adhere as well. When a solution containing 123 mg. of nitrogen as glycine, a relatively neutral amino acid, was added to a gram of resin, 50 per cent of the glycine was fixed to the resin. Likewise, when 157 mg. of nitrogen as a mixture of amino acids in solution (Amigen) were added to a gram of resin, 31 per cent of the amino acids was fixed to the resin. It must be

recognized that this type of experiment does not indicate the firmness of the bond between the amino acid and the resin.

In Vivo

These observations of the action of the resin suggested the alterations in electrolyte balance that might be anticipated *in vivo*. The actual alterations were measured following resin administration to a human subject.

The subject remained well and symptomless throughout the period of resin administration except for the last day when he was nauseated. He fully recovered in 24 hours. During the 80 days of resin administration the subject lost 3.6 kg. of weight which he regained by the ninth day after resin was discontinued. The mean urinary volume was greater during resin administration (2110 ml.) than during the control period (1640 ml.). The larger volume of urine excreted is for the most part accounted for by the extra water necessary to help swallow the capsules.

Urinary excretion of sodium averaged 109 mEq. per day during the control period. As the daily dose of resin was increased from 106 to 250 mEq., urinary sodium excretion fell to 10 mEq. per day (Figure 2). There were certain vagaries in urinary sodium excretion that corresponded with

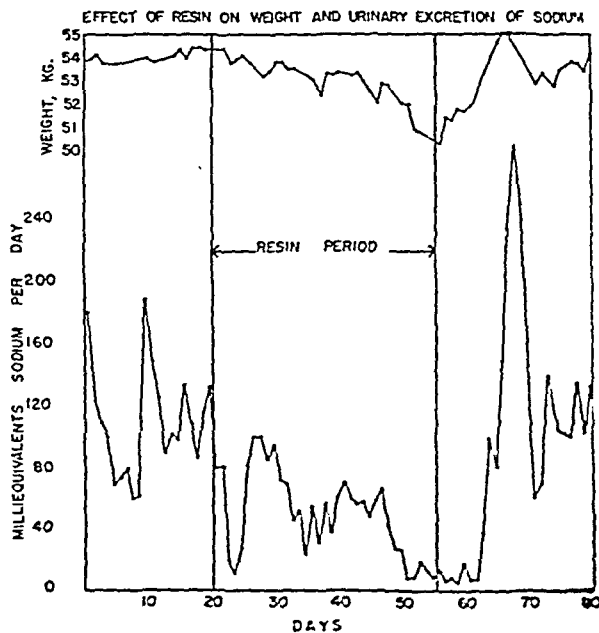


FIG. 2

TABLE II
Changes in plasma constituents during resin administration

Day	Period	Sodium	Potassium	Chloride	Carbon dioxide*	pH	Calcium	Phosphate	Alkaline phosph.	Urea nitrogen
		mEq./L.	mEq./L.	mEq./L.	mM/L.	units	mEq./L.	mM/L.	K-A units	mg. per cent
4	Control	140.0	4.68	102.8	27.	7.35	4.2	1.37	4.6	14.9
16	Control	140.0	4.16	101.0	32.4	7.45	5.9	0.88	8.6	9.0
20	Resin Introduced									
28	Resin	144.	3.63	104.5	27.4	7.45	5.8	0.98	3.0	16.8
37	Resin	137.2	3.61	101.0	25.2	7.38	5.3	0.88		13.2
46	Resin	140.8	4.20	108.0	21.3		5.15	0.95	6.8	13.5
53	Resin	135.3	2.65	112.0	18.9	7.45	4.9	0.92	4.4	13.7
55	Resin Discontinued									
56	Post Resin			109.0	22.5					
63	Post Resin	141.2	3.34	100.0		7.40	4.7	0.88	3.8	8.4
74	Post Resin	139.4	4.03	104.0	28.8	7.45	4.4	0.82		16.0

* Combining power

weight changes, the explanation of which is not clear. For ten days after the discontinuance of resin, it remained low (10 mEq. per day) and was then followed by a profuse diuresis of 325 mEq. per day before returning approximately to the level of the dietary intake. Plasma sodium did not vary appreciably during the study (Table II).

The patient experienced constipation towards the end of the resin period. This accounts for the low fecal excretion of sodium and potassium at this time (Figures 3 and 5). The feces for 13

was receiving 200 to 250 mEq. of resin per day, the fecal sodium representative of this period was about 80 per cent of the dietary sodium (Figure 3).

Urinary potassium excretion did not show the fluctuations that occurred in the urinary sodium excretion (Figure 4). From a mean control value of 78.5 mEq. per day it fell over a period of 30 days to a level of 7 mEq. per day as the dosage of resin increased. After the discontinuance of the resin the potassium excretion also remained at a low level for 10 days before it rose to equal the dietary intake. Plasma potassium remained relatively stable except for one value of 2.6 mEq. per liter toward the end of resin administration. Mean daily fecal potassium excretion increased from 5.8 mEq. during the control to 58.6 mEq. during resin feeding (calculated similarly to the sodium). Fecal potassium during resin administration was thus 68 per cent of the dietary potassium.

Mean daily urinary calcium excretion was 13 mEq. during the control period, 8.8 mEq. during resin administration and 11.2 mEq. in the post-resin period. The mean daily fecal calcium excretion was 40 mEq. during the control period and

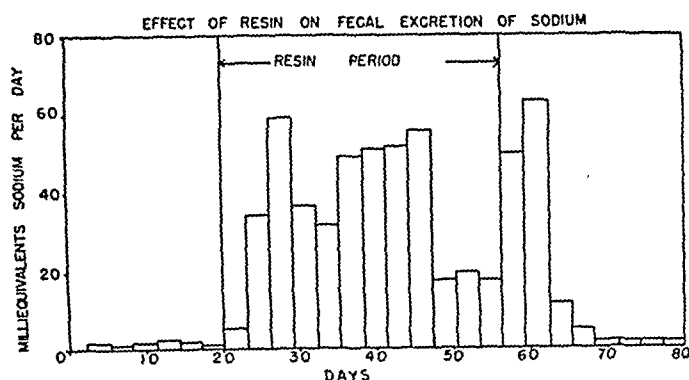


FIG. 3

days after the resin was discontinued contained normal amounts of nitrogen while the weight indicated that resin was still present. Consequently, feces excreted in these 13 days may be considered as part of the resin period. On this basis, the mean fecal excretion of sodium increased from 1.4 mEq. per day during the control to 51.0 mEq. per day during the resin period.² At the time the patient

²Liquonex CRW itself contains a small amount of sodium and the fecal excretion of sodium has been accordingly corrected.

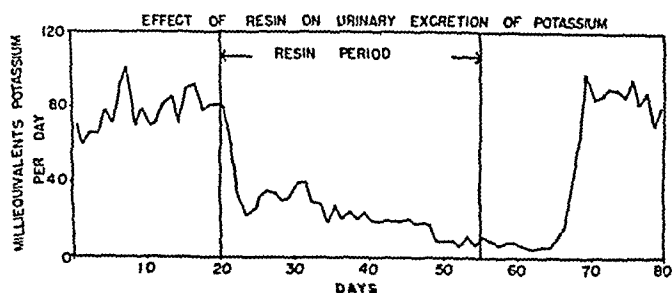


FIG. 4

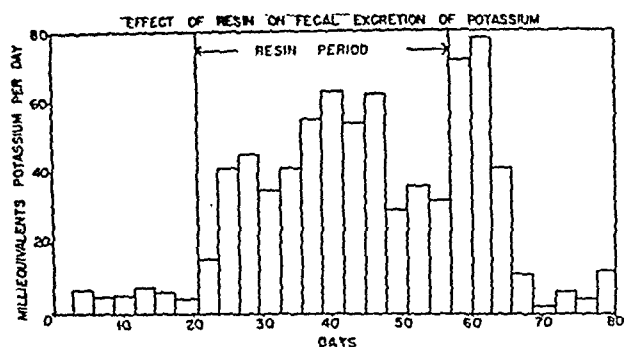


FIG. 5

35.1 mEq. during resin administration (Figure 6). Statistically the mean daily excretion of urinary or fecal calcium did not differ among these periods. There were no appreciable changes in plasma calcium or alkaline phosphatase.

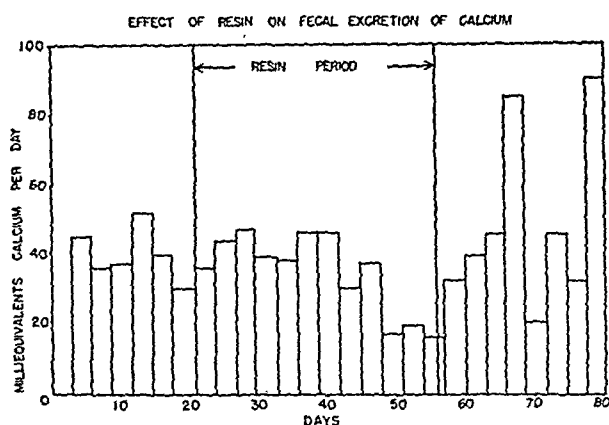


FIG. 6

During the control and resin periods dietary sodium, potassium and calcium compared well with the urinary plus fecal output of these ions. Balance studies thus failed to indicate loss of sodium, potassium or calcium from the body.

The mean daily urinary excretion of chloride, phosphate, and sulfate were not appreciably different in the control, resin and post-resin periods. They were respectively 124, 110 and 121 mEq. for chloride; 27.5, 29.7 and 22 mM for phosphate; and 32.3, 37.0, and 38.6 mEq. for sulfate. There were no appreciable changes in the fecal excretion of chloride or phosphorus. Plasma phosphorus was not altered.

The reduced absorption of sodium and potassium from the gut in the presence of a relatively constant absorption of chloride, phosphate, and

sulfate illustrates the mechanism of the acidosis induced by the resin. As resin administration progressed, plasma carbon dioxide combining power fell from 27 to 19 mM per liter and the chloride rose from 102 to 112 mEq. per liter with no significant change in pH of blood (Table II). This exemplifies the compensated metabolic acidosis described by Gamble (28).

The kidney balanced the excess chloride, phosphate and sulfate relative to sodium and potassium by formation of ammonia (Figure 7). Ammonia production increased from a mean value of 25 mEq. per day during the control period to 211 mEq. per day on the 34th day of resin administration. The mean urinary pH during the control periods was 6.3 and during resin feeding was 6.0. The mean daily titratable acidity increased from 17.2 mEq. during the control period to 25.6 mEq. during the resin period. It may be seen that the shift in pH did not contribute greatly to balance the anion excess. It is of interest that additional calcium was not excreted in the urine to help balance this anion excess.

URINARY ELECTROLYTE PATTERN DURING CONTROL AND RESIN PERIOD

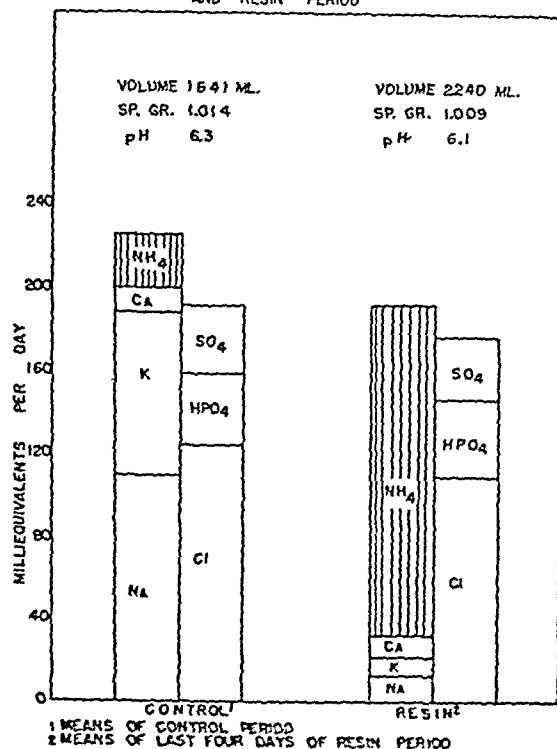


FIG. 7

The mean daily excretion of urinary nitrogen for control and resin periods was 10.2 and 10.5 grams respectively. In the first 10 days following discontinuance of the resin, urinary excretion of nitrogen fell to a daily mean of 8.8 grams following which the mean was 10.5 grams. The mean daily fecal nitrogen was 0.68, 0.68, and 0.61 grams for the control, resin and post-resin periods respectively. Except for the 10 days immediately after discontinuance of the resin, where there was a retention of nitrogen, total nitrogen intake of 11.9 grams compared well with total output. There was no consistent change in blood urea nitrogen.

Clinical Application

Two pertinent effects were noted in the administration of resin to this subject. Sodium was immobilized in the gut and a compensated metabolic acidosis was produced. These two situations would be acceptable in the therapy of edematous states. The resin was therefore tested for its usefulness in the therapy of two cases of congested heart failure and one case of cirrhosis of the liver with ascites and edema.

The patient A. C. was a 62 year old male with luetic heart disease and aortic insufficiency. He was observed for six weeks on a regular diet containing approximately 6 grams of salt. Moderate compensation was maintained with three 2 ml. injections of mercurhydrin each week plus 0.1 gram of digitalis daily. At the end of this six-week period mercurials were discontinued but the diet and digitalis were maintained. The patient was begun on gradually increasing doses of resin starting at 100 mEq. daily. After a slight weight gain during the first four days, his weight stabilized for the succeeding four days, whereupon he began to lose weight. As the dosage of resin was increased to 270 mEq. daily over a 16-day period, there was a 2.5 kg. weight loss. The resin was discontinued and he promptly gained 4.6 kg. in 10 days. The resin was again instituted and the patient lost 2.7 kg. in five days.

The patient C. G. was a 55 year old white female with rheumatic heart disease. During a control period of 37 days on an acid ash diet containing 3 grams of sodium chloride, she required 0.1 gram and 0.2 gram of digitalis every other day and 2 ml. mercupurin about every five days. Her greatest weight during this period was 46.9 kg., her lowest was 41.4 kg. With institution of resin in daily dosage of 230 mEq., diet was changed to one containing 6 grams of salt daily, mercupurin was discontinued, digitalis dosage was maintained. The patient's weight fell from 46.9 kg. to 42.2 kg. in 12 days. At this time the patient vomited. Digitalis toxicity was considered to be the cause and both digitalis and resin were discontinued. Serum carbon dioxide combining power was normal. Digitalis was resumed after five days in

daily dosage of 0.1 gram and the resin was resumed after 10 days. During the 10 days without the resin her weight increased from 42.2 kg. to 47.3 kg. With daily dosage of 150 to 200 mEq. of resin, she maintained a weight of 45.5 kg. over a 40-day period without requiring mercupurin. Plasma carbon dioxide combining power and plasma potassium were not altered in this period. When the resin was discontinued she gained 2 kg. in four days and experienced a return of paroxysmal nocturnal dyspnea.

The patient J. O. was a 52 year old white male with symptoms of decompensated cirrhosis of the liver with abdominal ascites and peripheral edema. During a seven-week observation period in the hospital, he accumulated fluid at a rate which required mercupurin every 10 days. His greatest weight was 59.4 kg., and his lowest, 56.0 kg. following mercupurin. With the institution of resin in dosage of 100 mEq. per day, his weight fell from 57.4 to 53.5 kg. in 12 days. The patient maintained his appetite fairly well during these 12 days but with the loss of edema fluid, cachexia became apparent. At this time plasma carbon dioxide content had fallen from 24.5 to 13.8 mM per liter, plasma chloride rose from 101 to 110 mEq. per liter and potassium rose from 2.9 to 5.9 mEq. per liter. Oral administration of the resin, however, was continued for another 10 days. There was a further weight loss to 49.5 kg. at which point ascites could not be detected clinically. The patient became anorexic. Plasma carbon dioxide content fell to 8.5 mM per liter, chloride rose to 114 mEq. per liter, potassium was 2.3 mEq. per liter. The resin was discontinued and alkalizing solutions containing potassium (15 mEq. per liter) were administered intravenously. Although the patient was cachectic and weak, he did not appear critically ill, when he suddenly developed an aberrant ventricular rhythm and expired.

DISCUSSION

Cation exchange resins taken orally reduce the absorption of sodium from the intestine. When an amount of resin sufficient to neutralize 150 mEq. of base was fed daily with a diet containing 100 mEq. of sodium (6 grams of salt), urinary excretion of sodium was reduced to 50 mEq. per day. Feeding 250 mEq. of resin with the same diet reduced daily urinary excretion of sodium to 8 mEq.

Salt restriction is, of course, important in the management of congestive heart failure. Its application, however, requires the patient to submit to the rigors of a selective diet. With the use of a cation exchange resin, the patient may satisfy his appetite within reasonable limits without absorbing excessive amounts of sodium. During periods of resin administration in two edematous patients

with heart failure there was a diuresis of existing depots of fluid and edema failed to reaccumulate.

In the case of cirrhosis of the liver, use of the resin orally was also successful in preventing the absorption of sodium from the gut. Since the most important part of the treatment of cirrhosis is a dietary regime high in protein, and since devising a high protein, low salt diet is difficult, the use of resin might be an important addition to therapy of this condition.

Unfortunately, sodium is not the only ion, but is, in fact, the ion least likely to occupy the resin when in competition with potassium or calcium. From the balance study it did not appear that the subject lost more potassium than he consumed. There was, however, very low urinary excretion of potassium for 10 days after the resin was discontinued. In view of the gain in weight of 3.6 kg. and the daily retention of 1.4 grams of nitrogen, during a period of low excretion of potassium and sodium it would appear that tissue and water were being replaced.

A calcium deficit would normally be anticipated from the *in vitro* observations, but did not occur in the balance study. The availability of calcium for absorption is closely related to the presence of insoluble complex acid radicals (*i.e.*, oxalates, phytates) (29). The resin produces, in effect, a markedly acid diet which would increase the availability of the insoluble calcium for absorption (29). This may or may not be the explanation, but the fact remains that the subject did not develop a detectable calcium deficiency.

The secretion of sodium, potassium and calcium into the lumen of the gut and their subsequent reabsorption creates an active interchange of electrolytes across the gut wall. Of the ions to which the resin is exposed in its passage down the gut, the amount of sodium is by far in excess of potassium or calcium. Thus, a larger portion of the resin was occupied by sodium *in vivo* than was anticipated by the studies *in vitro*.

Consideration must also be given to cations other than sodium, potassium, and calcium; in particular, magnesium, iron, manganese, copper and cobalt. Prolonged feeding of exchange substances may well lead to a deficiency of one or another of these cations. Since basic organic ma-

terials are also fixed to resin, these substances, too, must be considered. In the balance study, fecal nitrogen did not increase during resin feeding. It may be inferred from this fact that there were no excessive losses of amino acids in the stool. The losses of other basic organic substances (riboflavin, thiamin) were not measured. The magnitude of the problem of creating a deficiency as a result of the possible losses of these basic organic substances remains to be evaluated. The deficiency would depend greatly on the strength of the bond between the organic base and the particular exchanger.³

In exchange for cations, Liquonex CRW releases free hydrogen ion into the gut. Resin feeding is consequently equivalent not only to restricting salt intake but also to concomitantly administering 5–10 grams of ammonium chloride daily. With the usual administration of ammonium chloride, the kidney, in some measure, balances the anion excess with sodium and potassium. With the administration of resin, however, the availability of sodium and potassium for balance is small. In this instance, with the removal of sodium and potassium, ammonia production was forced to a maximum. It follows that administration of the resin to individuals with impaired renal function should be exercised with caution.

The disadvantage of oral administration of Liquonex CRW centered around the 20 to 25 size 00 capsules that had to be fed with each meal. Constipation may be experienced as the stool acquires the sandy consistency of the resin, but has so far only occurred in the subject of the balance study.

Evidence for calcium deficiency was lacking in this study but the possibility must still be considered. The evidence for potassium deficiency is stronger than for calcium and may well be a danger in the use of Liquonex CRW. Calcium and potassium occupy Liquonex CRW preferentially to sodium, an admirable characteristic for water softening for which the resin is primarily manufactured. This characteristic, however, is inherent in all cation exchange substances yet it does not

³ Patient C. G. has taken Liquonex CRW in daily dosage of 200 mEq. continuously for 10 months without clinical evidence of ill effects.

seem to invalidate its use for removal of sodium from the diet.

The limited clinical material presented here illustrates the advantages and dangers of administration of Liquonex CRW. The evident dangers are acidosis and potassium deficiency. It is far from clear how the acidosis might have contributed to the death of the patient with cirrhosis of the liver, but there may have been some indirect relationship. Excessive loss of potassium must also be considered although the electrocardiogram did not exhibit the expected pattern of potassium deficiency. The use of potassium lactate, or potassium bicarbonate in conjunction with the resin may prove to obviate these dangers without reducing the efficacy of sodium removal. Liquonex CRW, although representing an exchanger with one of the higher capacities, is by no means the ideal substance. Exchange substances of still higher capacity, exchanging ions other than hydrogen, if effective at the pH and electrolyte concentration of the intestine, would represent further advances in resin therapy.

CONCLUSIONS

1. The principle of the use of a cation exchange substance has been shown to be sound in retaining dietary sodium in the gastrointestinal tract to be excreted in the stool.

2. Its use in congestive heart failure and cirrhosis of the liver resulted in a diuresis of existing depots of fluid which did not reaccumulate while the resin was administered.

3. The undesirable characteristics of a particular exchange substance are indicated. With the use of Liquonex CRW, potassium depletion and acidosis must be considered as possible hazards.

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CARBON TETRACHLORIDE POISONING IN MAN.

I. THE MECHANISMS OF RENAL FAILURE AND RECOVERY¹

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Carbon tetrachloride poisoning in man, whether by inhalation or ingestion, is frequently associated with anatomical and clinical evidence of renal damage. Smetana (1) has shown that the distal tubule appears to bear the brunt of the renal pathological processes resulting in oliguria or anuria, and Woods (2) has demonstrated the identity of the renal pathology in this disease with that of the crush syndrome. This type of acute renal failure has therefore been included among the subclassifications of lower nephron nephrosis by Lucké (3). This author believes that the scant urine flow in this syndrome is adequately explained by the back-diffusion of the glomerular filtrate through the damaged tubular walls. This concept was first promulgated by Dunn, Haworth and Jones (4) working with experimental oxalate nephritis. Bywaters and Dible (5) concluded this to be the mechanism of anuria in the crush syndrome on the basis of morphology alone. A. N. Richards (6) was able to observe this phenomenon directly in the frog kidney poisoned with mercuric chloride. He noted the absence of urine formation in spite of normal or augmented glomerular filtration. Additional evidence cited for the occurrence of abnormal tubular permeability is the finding of negative T_{mD} 's³ by Bobey *et al.* (7) and of decreased creatinine/inulin clearance ratios by Richards, Westfall, and Bott (8) in experimental uranium poisoning in dogs; the demonstration of negative T_{mPAH} values by Redish, West, White-

head, and Chasis (9) in a child with oliguria due to sulfathiazole intoxication and by Marshall and Hoffman (10) in two patients with post-shock uremia; and the demonstration by Phillips and Hamilton (11) of markedly reduced renal creatinine extraction ratios in dogs with near normal renal blood flow following renal ischemia.

Recently attention has been directed toward the possible contribution of renal hemodynamic factors in the maintenance of the oliguria during the post-shock phase, when the full-blown picture of uremia becomes manifest. Trueta *et al.* (12) have demonstrated in man as well as other mammalian species the presence of juxtamedullary glomeruli with large efferent arterioles draining directly into the venous circulation through the vasa recta, thereby avoiding the usual peritubular capillary circuit. As an explanation for the oliguria in the crush syndrome these authors postulate a neurogenic mechanism resulting in the shunting of blood from the active filtering and secreting cortical zone to the poorly functioning juxtamedullary region of the kidney.

Phillips and Hamilton (11) demonstrated rapid recovery of renal blood flow, as measured by the Fick principle, in dogs following two hours of complete renal ischemia. It is unfortunate that they did not perform renal blood flow studies on their dogs with three and four hours of renal ischemia, since it was these animals which subsequently developed progressive uremia, thus more nearly simulating the human material. Bobey *et al.* (7) observed immediate and maintained normal renal blood flow values in dogs with uranium poisoning for periods as long as 14 months. However, since uranium exerts its maximum toxic effects upon the proximal tubule (13) and the dose used was insufficient to cause oliguria, neither the histological nor usual clinical manifestations of the human syndrome under discussion were simulated in this series of experiments.

Corcoran, Taylor and Page (14) performed serial inulin and diodrast clearances upon one pa-

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³ Abbreviations used will be as follows: D = diodrast, IN = inulin, CR = endogenous creatinine, PAH = p-aminohippurate, C = clearance, thus C_{IN} is clearance of inulin, T_{mPAH} and T_{mD} = tubular maximal excretory capacity for p-aminohippurate and diodrast respectively. E = renal extraction ratio, RBF = renal blood flow, U/P ratio = ratio of urine concentration to plasma concentration.

ith carbon tetrachloride poisoning during every phase and found values for C_{IN} , C_D , C_{PAH} of 5.8 cc./min., 67.8 cc./min. and 3.7 in. respectively on the 10th day of the

By the 64th day these values had returned to the normal range. On the basis of the data these authors concluded that in ad-tubular back-diffusion depressed renal v, caused by renal swelling, plays an im-ple in the production of oliguria. Mar-

Hoffman (10), using mannitol and formed similar studies upon two cases

back uremia and one case of carbon tetrachloride poisoning. The clearance values were all markedly depressed during the early recovery phases with a gradual return to normal. Burnett *et al.* (15) studied renal clearances in wounded soldiers. They found significant depressions of mannitol and PAH clearances and Tm_{PAH} in those patients who had suffered moderate to severe shock even though the studies were undertaken from 14 hours to 30 days after recovery from shock.

With the severe tubular damage and probable tubular back-diffusion of the test substances that occur during the early recovery phase of this syndrome it would seem that the estimation of hemodynamic factors on the basis of uncorrected clearance measurements yields data of dubious significance. Certainly when a marked decrease in the renal extraction of PAH occurs, as has been adequately shown to be the case following total renal ischemia in the dog by Selkurt (16) and Phillips and Hamilton (11), the clearance of this substance cannot be used as a significant measure of effective renal plasma flow. These limitations of the clearance methods were early emphasized by Smith (17).

We have therefore studied the renal extraction ratios of PAH and inulin together with their clearances and other aspects of renal function in four patients with acute carbon tetrachloride poisoning during various stages of the disease. The results indicate that a marked reduction in renal blood flow is in fact important in the maintenance of the oliguria, if not in its initiation.

METHODS

Studies of renal function were performed on four male patients with acute renal damage due to inhalation

of carbon tetrachloride.⁴ These studies were started between the eighth and 11th day from the onset of oliguria and continued periodically for as long as 60 to 320 days. Simultaneous inulin, p-aminohippuric acid, urea, endogenous creatinine and chloride clearances and the measurement of Tm_{PAH} were performed according to the standardized techniques of Smith, Goldring, and Chasis (18). The technique of Warren, Brannon, and Merrill (19) and Bradley and Bradley (20) was utilized for the renal vein catheterizations in order to determine the renal extraction of PAH, inulin and oxygen.⁵ Renal blood flow was calculated by the formula $RBF = (UV/A-R) \times (1.00/1.00-H)$, where A, R, and U represent arterial, renal venous and urine concentrations respectively of PAH or inulin in mgm./cc., V represents urine flow in cc./min., and H is the hematocrit expressed as a fraction of 1.00. Since repetition of renal vein catheterization in any one individual during the early recovery phase was thought unwarranted this procedure was performed once on each individual at times representing different phases of the recovery process. In each case the final position of the catheter was checked by means of roentgenograms.

On the days intervening between the standard clearance studies, when the 24 hour urine flow exceeded 1000 cc., endogenous creatinine clearances alone were obtained using two to four hour periods and voided urine specimens. One blood specimen was drawn at the midpoint of the urine collection period. In two of the subjects with severe oliguria, inulin and PAH clearances were determined, each on one occasion, by giving one intravenous injection which acted as both the priming and sustaining doses. Blood was drawn approximately every six hours and urine was collected at the end of a 24 hour period by catheterization and bladder washout. Total PAH (free and acetylated) and inulin analyses were performed on these specimens. The urine of the preceding day was used for the determination of the rate of excretion of the inulin blank (UV_0).

The other studies of renal function consisted of the usual clinical measurements for urine concentrating capacity, urinary acidity, efficiency of phenol red excretion, examination of the urinary sediment, plasma creatinine and urea nitrogen determinations and the semi-quantitative estimation of albuminuria.

Inulin was determined by a modification of Harrison's method as described by Goldring and Chasis (21), with the exception that 2 cc. of undiluted plasma were added to 6 cc. of 20 per cent yeast and 1.0 N NaOH was substituted for 1.1 N NaOH in the precipitation of proteins. PAH, both free and total, was determined by the method of Smith *et al.* (22); creatinine by the Brod and Sirota modification of the Bonsnes and Taussky method (23);

⁴ These patients were on the 1st and 2nd Medical Services of Mt. Sinai Hospital. The clinical details will be presented elsewhere.

⁵ Drs. Sigmund Brahm and Irving Kroop collaborated with the author in the performance of the renal vein catheterizations.

TABLE I
The renal extraction ratios of PAH and inulin and renal plasma flow
in four cases of carbon tetrachloride poisoning

Patient	Number of days of oliguria	Day from onset of oliguria	Renal extraction ratio $\frac{A-R}{A}$		Renal plasma flow*		Renal blood flow	$1.08 \times C_{PAH}$	C_{IN}
			PAH	Inulin	$\frac{PAH}{UV} \frac{UV}{A-R}$	$\frac{Inulin}{UV} \frac{UV}{A-R}$			
F. M.	10	8	0.034	0.00	cc./min. 24.5	cc./min. 0	cc./min. 40.8	cc./min. 0.944	cc./min. 0.00
F. H.	2**	13	0.106	0.045	160	167	232	21.4	7.84
H. Z.	11	24	0.895	0.133	640	708	896	618	97.2
W. M.	17	37	0.594	0.149	592	434	897	392	56.0

* U, A and R represent urine, femoral arterial and renal venous plasma concentrations of PAH or inulin respectively, in mgm./cc., and V represents urine flow in cc./min. These values were obtained from averages of three extraction ratios and three urine collection periods, except for F. M. in whom one urine collection period was used.

** The occurrence of oliguria is questionable in this case because of difficult anamnesis. Its duration, if present, was definitely no longer than two days.

blood oxygen by the method of Roughton and Scholander (24); chlorides by Van Slyke and Hiller's adaptation of Sendroy's method (25); and urea by the aeration technique of Van Slyke and Cullen (26). All colorimetric determinations were performed on the Coleman Jr. spectrophotometer.

RESULTS

1. Renal extraction ratios and renal blood flow

Control PAH renal extraction ratios performed on four patients without renal disease yielded values of 0.904, 0.890, 0.910 and 0.895. The values for PAH and inulin extraction ratios and the renal plasma flows calculated therefrom in the four patients with carbon tetrachloride poisoning are presented in Table I. It is evident that during late oliguria and early diuresis there was a marked reduction in renal blood flow, and that the perfused tissue extracted PAH with poor efficiency, so that $1.08 \times C_{PAH}$ yielded values much lower than $UV/A-R$ for PAH, the true renal plasma flow.⁶ Thus,

⁶ $\frac{UV}{A-R}$ represents the application of the Fick principle to the determination of renal plasma flow, and as long as a measurable quantity of PAH is present in the urine it is an expression of true renal plasma flow, being independent of the efficiency with which the tubules extract PAH from the plasma. C_{PAH} , the clearance of PAH, or $\frac{UV}{A}$ in the normal kidney is a measure of renal plasma flow to effective functioning renal parenchyma and is therefore dependent upon the efficiency of renal extraction. It normally is about 92 per cent of the total plasma flow, the remaining 8 per cent representing plasma flowing to non-functioning tissue such as capsule, interstitium and perirenal fat. Hence $1.08 \times C_{PAH}$ should

patient F. M. on the eighth day following the onset of oliguria had a renal plasma flow of only 24.5 cc./min. and his kidney extracted PAH with only 3.4 per cent efficiency.⁷ The value for $1.08 \times C_{PAH}$ was reduced to 0.94 cc./min. Patient F. H. on the 13th day had a renal plasma flow of 160 cc./min. in spite of a diuresis of over 3000 cc. in 24 hours. His PAH renal extraction efficiency was 10.6 per cent; $1.08 \times C_{PAH}$ was 21.4 cc./min. Patient H. Z. had both a normal renal plasma flow and PAH extraction ratio by the 24th day, so that $1.08 \times C_{PAH}$ closely approximated the true renal plasma flow. In contrast, W. M., who suffered oliguria for 17 days, still had a reduced PAH extraction (59.4 per cent) on the 37th day even though his renal plasma flow was normal (592 cc./min.). The low renal blood flow figures of 896 and 897 cc./min. for H. Z. and W. M. respectively are merely expressions of low hematocrits.

The use of the inulin extraction ratio for the calculation of renal plasma flow is fraught with error because of the comparatively low arterio- approximate $\frac{UV}{A-R}$ closely. A discrepancy between these values merely reflects the deviation of renal extraction of PAH from the average 92 per cent value.

⁷ Technical errors in the withdrawal of renal venous blood, such as too much suction causing the admixture of inferior vena caval blood with renal venous blood, would yield an erroneously low extraction ratio. However, this would also yield an erroneously high plasma flow, since the value $A-R$ in $\frac{UV}{A-R}$ would be smaller than the true value.

CARBON TETRACHLORIDE POISONING IN MAN

1415

TABLE II
Serial renal clearance studies in four patients recovering from carbon tetrachloride poisoning*

TABLE II

Serial renal clearance studies in four patients recovering from carbon tetrachloride poisoning*

Patient	Number of days of oliguria	Day from onset of oliguria	Urine flow cc./24 hrs.	Plasma clearances				Clearance ratios			U/P ratios		Effective renal blood flow cc./min.	TmPAH mgm./min.	Filtration fraction	C _{IN} TmPAH	C _{PAH} TmPAH	Plasma creat. mgm./100 cc.	Plasma urea N mgm./100 cc.
				Inulin	Creat.	Urea	PAH	Creat. In.	In. Urea	Inulin	Chloride								
				cc./min.	cc./min.	cc./min.	cc./min.												
F. M.	10	8**	240	0.00						0.00	0.71							22.4	58.0
		10	1500		0.79	0.35	0.81				0.87							24.6	123
		11	2010		0.28	0.16					0.19							26.5	128
		17	1440		3.79	2.28					0.09							11.7	106
		20	1592		10.4	6.48					0.37							6.00	78.0
		23	1830		23.6	11.6					0.40		568	33.3	0.106	1.30		2.80	101
F. H.	2(?)	26	2715	43.5	52.2	31.5	410	1.20	1.38	18.7	0.35		592	52.6	0.127	0.96		2.00	29.0
		31	1790	50.6	60.8			1.26		10.3			854	71.3	0.145	1.04		1.68	18.0
		38		74.0	81.0			1.10									1.52		
		60															1.20		
		10	2290	3.78	4.34	4.00	14.4	1.15	0.944	2.61	0.21		1.85	0.261	1.94	7.82	16.2	146	
		12†	3700	3.55	3.95	2.62	17.4	1.11	1.16	2.51	0.20		0.389				16.9	166	
H. Z.	11	13†	3370	6.74	7.26			1.06		3.24	0.26			0.223					
		21	2250	37.7	38.3	20.5	169	1.02	1.84	14.6	0.35		532	17.0	2.22				
		34		57.3	83.8	53.0	370	1.46	1.08	8.94			600	51.6	1.11	9.96	3.70	81.0	
		47		70.1	90.3			1.11		9.10			627	72.2	0.155	7.16	1.32	20.0	
		61		78.2	93.0	390	389	1.25		15.2			669	83.9	0.201	5.40	1.24		
		132		90.3				1.43	1.39	14.0			980	76.2	0.232	4.64	1.16		
W. M.	17	220		110	158			1.12					816	0.190	1.18			14.0	
		307		117	131	84.2	502			0.47			78.4	0.232	1.49			13.0	
		10	880							0.66							1.16		
		13	1180		1.42	1.54				0.08							0.99	14.0	
		18	1965		4.50					0.18									
		20	1460		14.1					0.30									
Normal Values	17	21	2575	86.5	73.2	510	0.92	1.97	74.3					0.170					
		24	1311		8.54														
		29	3915		46.3														
		35																	
		106	94.4	99.7	89.9	433	0.96												
		250	97.0	107	473	603	1.07												
Normal Values	17	95	0.49	0.48	0.14	2.38	0.97	3.43	7.50					0.208					
		19	2272	1.66		9.91	2.44							0.167					
		26	2378	25.2	31.0	143	2.44							0.176					
		37	2975	57.6	78.6	362	1.23	1.38	12.3	0.54				0.152					
		48		71.0	85.6	452	1.37	1.18	17.3	0.20				0.165					
		63		74.6	89.0	570	1.20	1.18	43.1	0.20				0.205					
Normal Values	17	96	91.0	100	470	538	1.19							0.218					
		175	97.2	104	618		1.10							0.165					
		331	108	117			1.04							0.205					
														0.205					
														0.205					
														0.205					
Normal Values				131±21.5	697±136	77.5±13													

* All clearance figures represent the average of three clearance periods and are corrected for a body surface of 1.73 m².

** These clearance figures were of the 24 hour type, as explained under methods.

† The plasma and urine blanks for inulin on these days were assumed to be the same.

*All clearance figures represent the average of three clearance periods and are corrected for a body surface area of 1.73 sq. m.
 **These clearances were of the 24 hour type, as explained under methods.
 †The plasma and urine blanks for inulin on these days were assumed to be the same as those found on the 10th day.

venous difference, high plasma and urine inulinoid blanks and the relative insensitivity of the analytical method as compared to the PAH method. The good check in the calculations of the renal plasma flow by the two methods in patient F. H. was surprising. The poorer checks obtained in the other three subjects were expected. The C_{IN} and E_{IN} of 0.00 may be due to lack of filtration or tubular back-diffusion of the inulin molecule or a combination of both.

A urea extraction ratio determined in F. M. simultaneously with PAH yielded a value of 0.149 and a calculated renal plasma flow of 24.1 cc./min., affording an excellent check on the figure 24.5 cc./min. as obtained by means of the PAH extraction ratio.

A renal arteriovenous oxygen difference of 3.0 volumes per cent with an oxygen utilization of 26.8 cc./min. obtained in subject W. M. on the 17th day are within the normal range. The oxygen utilization was not determined in the other three patients.

2. Renal clearance studies

The results of the renal clearance studies are presented in Table II. There was a marked reduction in the clearance values of all the substances studied during oliguria and early diuresis. The following points are of special interest:

a. With the onset of diuresis the increment in the clearances of inulin, creatinine or urea were extremely small or even in the negative direction in patients F. M. and F. H. on the 10th and 12th days respectively. This suggests that the initial diuresis may be a reflection of tubular repair rather than an increase in glomerular filtration. However, since active water reabsorption appears to be markedly impaired at this stage, an increase in glomerular filtration, *per se*, of a magnitude small enough to be undetected with present methods, may result in a large increment in daily urinary output.

b. The U/P ratio for chlorides was always significantly less than 1.00, indicating preservation of selective tubular reabsorption of chlorides. During early diuresis the U/P ratio for inulin reached minimum values of 1.38 and 2.51 in patients W. M. and F. H. respectively, with simultaneous chloride U/P ratios of 0.51 and 0.20.

Since the kidney is unable to do osmotic work at this stage, the urine being isosmotic with plasma (sp. gr. 1.010), water must follow the actively reabsorbed chloride in order that osmotic equilibrium be maintained. Such low inulin U/P ratios, therefore, in the presence of considerable selective chloride reabsorption, is suggestive of abnormal back-diffusion of inulin.

c. The clearance of PAH, though reaching very low figures during the early recovery phase, was always greater than C_{IN} and C_{CR} . In addition, Tm_{PAH} values were never negative, indicating continued active tubular secretion of PAH.

d. In patient H. Z., who sustained the least renal damage as judged by recovery rate, the CR/IN clearance ratio remained close to unity between the 24th and 250th days. The other three patients revealed high CR/IN clearance ratios during the early recovery period with gradual declines towards unity as recovery neared completion.

3. Recovery of function

The recovery of the inulin, creatinine and PAH clearances and the Tm_{PAH} values together with declining plasma creatinine levels are plotted against days following the onset of oliguria for the individual patients in Figure 1.

From Figure 1 it is evident that the recovery process may be arbitrarily divided into three functional phases. The first phase was initiated with the onset of diuresis and lasted from one to eight days. In spite of diuresis the plasma creatinine concentration continued to rise for one to three days apparently because of the absence of or very slight improvement in the clearance values. The second phase, starting between the second and ninth days, was initiated by a rapid fall in plasma creatinine concentration and a rapid increase in all the clearance values, reaching 40 to 70 per cent of the expected norm by the 40th day from the onset of oliguria. During this phase the relative C_{CR} increment was greater than the C_{PAH} , which in turn was slightly greater than the C_{IN} increment. The third phase, starting from about the 40th day was associated with gradual rises in C_{IN} , C_{PAH} and C_{CR} , the lower limit of normal (80 per cent of the statistical norm) being reached between the 100th and 200th day. Except for one C_{CR} value in patient F. H. on the 220th day the clearance values remained well under 100 per

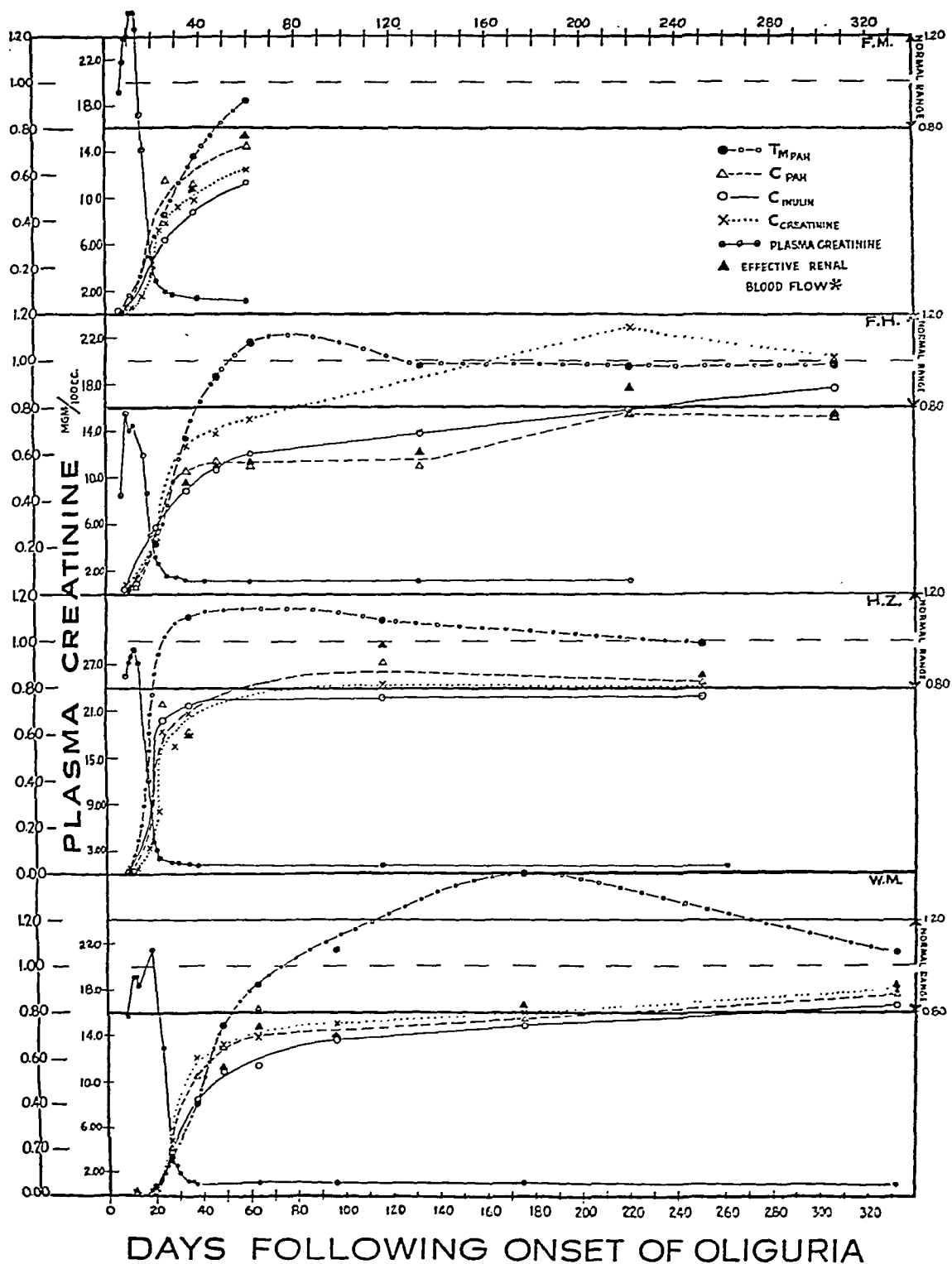


FIG. 1. INDIVIDUAL RECOVERY CURVES FOR C_{IN} , C_{CR} , C_{PAH} , T_{MPAH} , AND PLASMA CREATININE CONCENTRATIONS IN FOUR SUBJECTS RECOVERING FROM CARBON TETRACHLORIDE POISONING

Cessation of oliguria occurred in F. M. on the 10th day, in F. H. on the third day, in H. Z. on the 11th day and in W. M. on the 17th day.

* Effective renal blood flow, calculated by $\frac{C_{PAH}}{1-H}$ where H is the hematocrit expressed as a fraction of 1.00, is not recorded before the 30th day because of poor renal PAH extractions.

cent of the statistical norm throughout the period of observation. Patient W. M., who suffered the most severe functional renal damage with oliguria for 17 days, had a C_{IN} of 108 cc./min. and a C_{PAH} of 618 cc./min. on the 331st day from the onset of oliguria.

In marked contrast, Tm_{PAH} for all four patients rapidly reached high normal values in the third phase of recovery, with patient W. M. obtaining the supernormal value of 130 mgm./min. per 1.73 sq. m. by the 175th day. With the further passage of time all the Tm_{PAH} values declined, leveling off at the expected normal value in F. H. by the 132nd day, in H. Z. by the 250th day and in W. M. by the 331st day. Because of the continued relative depression of C_{PAH} and C_{IN} the ratios C_{IN}/Tm_{PAH} and C_{PAH}/Tm_{PAH} in all four subjects remained lower than normal.

There was no correlation between the speed of recovery and the number of days of oliguria or the maximum plasma concentration of creatinine or urea. Thus, patient H. Z., the most critically ill of the four because of severe pulmonary edema, who suffered marked oliguria for 11 days and had peak plasma concentrations for creatinine and urea nitrogen of 32.0 and 226 mgm./100 cc. respec-

tively, recovered most rapidly. Conversely, although F. H. had questionable oliguria for two days the renal damage sustained by him was as severe as the two with oliguria 10 and 11 days.

The sequence in which the usual clinical tests of renal function returned to normal is presented in Table III. The relatively low peak values for plasma urea nitrogen and creatinine concentrations in patient W. M. in spite of 17 days of severe oliguria is due to two runs on the artificial kidney.⁸ The time of return to normal values of the plasma urea nitrogen concentration (8 to 18 mgm./100 cc.) was from the 24th to the 36th day from the onset of oliguria; for the plasma creatinine concentration (0.80 to 1.20 mgm./100 cc.) from the 29th to the 61st day.

All four patients had proteinuria. During oliguria the urine protein concentration was roughly between 1.5 to 3.0 gms./1000 cc. in three patients. W. M. spilled about 3 to 6 gms./1000

⁸ Drs. H. E. Leiter and I. G. Kroop placed this patient on the Kolff artificial kidney for six hours on the ninth day, during which time his plasma urea nitrogen concentration dropped from 108 to 69 mgm./100 cc. A second dialysis on the 15th day caused a drop from 98 to 57 mgm./100 cc.

TABLE III
The sequence in which the clinical tests of renal function returned to normal in four cases of carbon tetrachloride poisoning

Patient	Number of days of oliguria	Plasma urea nitrogen			Plasma creatinine			Proteinuria		Specific gravity		Phenol red excretion		Day of disappearance of abnormal urine sediment
		Maximum conc.	Day of max. conc.	Day of return to normal*	Maximum conc.	Day of max. conc.	Day of return to normal*	Severity	Duration	Duration of fixation	12 hour water deprivation	Day	Per cent of 6 mgm. in 2 hours	
		mgm./100 cc.			mgm./100 cc.				days	days	day value			
F. M.	10	131	15	30	26.5	11	40	1-2+	30	22	33 1.018	25 33 60	38 45 75	20
F. H.	2**	166	12	26	16.9	12	61	1-2+	30	26	27 1.011 132 1.019 220 1.025	23 28	41 70	20
H. Z.	11	226	14	24	32.0	13	29	1-2+	29	20	31 1.012 106 1.020 252 1.020	30	55	26
W. M.	17	108	8	36	20.9	19	37 •	2-4+	37	35	33 1.014 96 1.025 175 1.026	33	70	23

* Arbitrary maximum normal values for plasma urea nitrogen and creatinine concentrations were chosen as 18 and 1.20 mgm./100 cc. respectively.

** The occurrence of oliguria is questionable in this case because of difficult anamnesis. Its duration, if present, was definitely no longer than two days.

cc. during this stage. With the onset of diuresis the daily proteinuria gradually decreased in quantity so that by the 30th day it had disappeared in all except W. M. In the latter it did so by the 37th day. All four exhibited occasional faint traces of proteinuria for two to three months thereafter with eventual complete subsidence.

Following the onset of oliguria the urinary specific gravity gradually became fixed between 1.009 and 1.011 in all four patients and remained so for 20 to 35 days. The ability to concentrate urine to a minimum specific gravity of 1.025 after a 12 hour water fast returned relatively late, varying between 96 and 220 days.

The ability to secrete an acid urine was only slightly impaired from the onset of oliguria in three of the subjects, who had urine pH's ranging from 5.4 to 6.7. In patient W. M., however, up to the 13th day urine with an average pH of about 6.0 was passed. From the 13th through the 23rd day the urine pH ranged between 7.0 and 8.0 in spite of mild clinical acidosis. Acid urine was again excreted on the 24th day when urine with a pH of 5.5 was passed.

DISCUSSION

The marked reduction of renal blood flow during late oliguria and the early recovery phase in carbon tetrachloride poisoning may be a sequela rather than the initial cause of the renal failure. Adequate explanation for this depression of blood flow is found in the development of severe interstitial edema and inflammatory swelling of the kidneys as described by MacMahon and Weiss (27), Smetana (1), Woods (2) and Lucké (3). Unfortunately, because of the nature of the methods, it has not been possible to measure renal blood flow in man at the onset of oliguria. The experimental results of Richards (6) and Bobey *et al.* (7) with nephrotoxins suggest that renal blood flow may be normal during the initial phase of acute renal failure.

The morphological and clinical similarities of the post-shock and the nephrotoxic anurias have been well established (2, 3). The common denominator appears to be tubular injury. The occurrence in man of severe and prolonged renal anoxia during shock has been demonstrated by Lauson, Bradley, and Cournand (28), and Badenoch and Darmady (29) found identical tubular

lesions in men who died of post-shock uremia and in rabbits which developed uremia following two-hour ligation of the renal arteries. Thus, prolonged renal anoxia simulates the nephrotoxic action of carbon tetrachloride.

On the basis of the available information it is possible to formulate a working hypothesis concerning the pathogenesis of anuria and the mechanism of recovery from the acute renal failure of carbon tetrachloride poisoning. During the first 24 to 48 hours after exposure, because of nausea and vomiting, dehydration may be present. During this phase of gastro-intestinal irritation the urine, although scant, may be of normal composition and of high specific gravity. The tubules, still intact functionally, respond to the needs of the organism by maximally reabsorbing water. Between the first and third days after exposure the nephrotoxic action becomes manifest with severe oliguria or anuria and rapidly rising plasma urea and creatinine concentrations. Because of abnormal glomerular permeability considerable protein and gross blood may be present in the scant urine that is passed. Until studies of *early* anurias are performed in man we must tentatively accept the findings of the experimentalists and explain the scant urine flow of this stage by back-diffusion of the glomerular filtrate across the damaged tubule wall. "The organism urinates into the kidney instead of the bladder."² There is now convincing evidence to minimize the role of pigmented casts in the production of oliguria by mechanical blockage (3).

As oliguria progresses renal blood flow becomes markedly diminished. The resultant anoxia added to the initial toxic insult causes maximal depression of tubular function. Some of the small amount of filtrate now formed appears to be lost by back-diffusion. It is of some interest that during this phase and that of *early* diuresis, although there is fixation of urinary specific gravity and markedly depressed phenol red excretion, TM_{PAH} and E_{PAH} , the ability to retain chloride and to secrete an acid urine may be only slightly diminished.

Tests used for the secretory activity of the proximal tubular system during this phase may yield erroneously low values because of back-diffusion through the severely disorganized distal tubular

² This simile originated with Dr. Paul Klemperer.

system. Apparently carbon tetrachloride poisoning causes less injury to the proximal tubules than other circumstances leading to acute renal failure, as in all the published cases in which functional studies were performed (six including the four presented herein) Tm_{PAH} was never negative and returned to normal values with relative rapidity.

Diuresis initiates the recovery process. It occurs without significant changes in the clearance values and in the presence of low renal blood flow, and probably represents reestablishment of the tubular barrier to the indiscriminate back-diffusion of the filtrate. The rapid increase in tubular and glomerular function of the second stage of recovery is the result of tubular regeneration, diminishing renal interstitial pressure and rising renal blood flow. The gradual increase in filtration and blood flow of the third stage of recovery probably represents gradual reabsorption of inflammatory tissue and reestablishment of the finer vascular channels. Burwell, Kinney, and Finch (30) present autopsy findings of a case of renal injury three months after an episode of intravascular hemolysis, with death due to an unrelated cause, revealing cortical scarring. In a patient with acute carbon tetrachloride poisoning causing four days of oliguria who died 10 months later from an unrelated cause, Simon (31) failed to demonstrate significant renal pathology.

The high values for Tm_{PAH} obtained soon after the 40th day may represent transient compensatory hyperfunction of the proximal tubular system. The ability to concentrate urine maximally is the last tubular function to return to normal, probably representing final complete functional integrity of the distal tubule. These functional aspects of tubular recovery reflect the morphological pattern of mild proximal tubular damage and severe disorganization of the distal tubules.

Since a severe absolute reduction in renal blood flow has been demonstrated in the late oliguria of carbon tetrachloride poisoning, the shunt described by Trueta and his colleagues, if operative at the time of measurement, could not have been of great functional significance, since such a mechanism implies little reduction in total renal blood flow, but merely cortical bypassage. There has been no evidence to show that such a mechanism is responsible for the initiation of anuria in man.

All data so far available indicate that this juxta-medullary bypass is of little physiological import in normal man (32, 33), in man with increased intra-abdominal pressure (20), in essential hypertension (34) and in congestive heart failure (32, 35) by the demonstration of continued normal renal extraction of PAH in these conditions.

CONCLUSIONS

1. By means of the Fick principle the renal plasma flow was determined in four male subjects with acute carbon tetrachloride poisoning. Renal venous blood was obtained by catheterization of the right renal vein. P-aminohippurate was used as the test substance. In two of the subjects there was a marked reduction in the renal plasma flow and PAH renal extraction ratio. On the eighth day of oliguria the renal plasma flow in one of these subjects was 2.45 cc./min. and the PAH extraction ratio 0.034. On the 13th day from the onset of a questionable two-day oliguric period the second subject had a renal plasma flow of 160 cc./min. and a PAH extraction ratio of 0.106 in spite of a urine flow of over 3000 cc./24 hrs. In a third subject both the renal plasma flow and the PAH extraction ratio were normal on the 24th day. The fourth subject, who had suffered oliguria for 17 days, had a renal plasma flow of 592 cc./min. on the 37th day and a continued depression of the PAH extraction ratio to 0.594. Because of low PAH extraction ratios C_{PAH} is not a valid measure of renal plasma flow during oliguria and early diuresis following carbon tetrachloride poisoning.

2. The oliguria and anuria of carbon tetrachloride poisoning and the markedly depressed renal clearances of all substances during early diuresis are the results of a marked reduction in renal blood flow and glomerular filtration as well as abnormal tubular back-diffusion of the filtrate. It is probable that back-diffusion plays the most important role during early oliguria as decreased renal blood flow does during late oliguria and early diuresis.

3. The recovery of renal function following acute renal failure due to carbon tetrachloride poisoning is characterized by three clinical phases. The first phase starts with the cessation of oliguria and is associated with rising plasma creatinine and urea concentrations in spite of an adequate urine flow. It lasts from one to three days, during which

time there is little change in C_{IN} , C_{CR} and C_{PAH} . The second phase starts with a rapid decline in the plasma urea and creatinine levels and a simultaneous rapid rise in C_{IN} , C_{CR} and C_{PAH} which reach 40 to 70 per cent of normal by the 40th day from the onset of oliguria. The third phase, starting about the 40th day, is characterized by gradual improvement in renal blood flow and glomerular filtration, so that the lower limit of normal is reached between the 100th and 200th day. During this phase the ability to elaborate a maximally concentrated urine is recovered.

4. During the early part of recovery phases Tm_{PAH} reached high normal or supernormal values in three of the patients, ranging between 83.9 and 130 mgm./min. per 1.73 sq.m. These gradually declined to the expected norm. The ratios C_{IN}/Tm_{PAH} and C_{PAH}/Tm_{PAH} , however, were significantly lower than normal in all four patients when last determined on the 60th, 250th, 307th and 331st day, respectively, suggesting some residual vascular damage.

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NORMAL RESPIRATORY AND CIRCULATORY PATHWAYS OF ADAPTATION IN EXERCISE¹

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Rahn and Otis (1) have described the normal changes in the alveolar gases during work and demonstrated the quantitative relationships between alveolar oxygen, carbon dioxide, respiratory quotient and ventilation by means of continuous recording of gas analyses. They were able to delineate the pathways of change which are controlled by the excretion of carbon dioxide, and observe the effects of hyperpnea, hypoxia, hypoventilation and CO₂ breathing as well. Pelnar (2), at first independently and later in conjunction with Rahn, observed the changes in expired air composition in normal subjects as well as in patients with cardiorespiratory impairment in relation to work. By means of a simple diagram relating the changes in O₂ and CO₂ content of expired air with the respiratory quotient during the stress of exercise, he felt that he could delineate respiratory function better than by any other means in a manner that paralleled the intensity of dyspnea in patients. Between the extremes of *a*) normal performance with a large circular curve and *b*) the abnormal performance with no change in these values during exercise, Pelnar found a series of results which were characteristic of the varied stages of dyspnea. Thus he was able to evaluate functional performance largely in terms of changes in the respiratory gases during exercise. In contrast to this are the older methods reported by Kaltreider and McCann (3) who relied chiefly on ventilation volumes and ventilation indices to appraise pulmonary capacity during work. Both Rahn and Otis (1) and Pelnar (2) express the

view that the level of ventilation is controlled in large measure by the CO₂ when breathing normal ambient air in which the strong stimulus of hypoxia is lacking. The hypoxic stimulus of breathing 15% oxygen in normals or hypoxemia from pulmonary disease in patients clearly induces greater ventilation levels, however.

With these considerations in mind we have attempted to repeat these studies in both normal subjects and patients with disease to ascertain not only the normal respiratory pathways, but also the circulatory pathways during exercise and recovery. Thus the inter-relationships between the two can be considered in a way to demonstrate how the adaptations of each complement and spare the other. The techniques employed consist essentially of controlled work-loads by means of walking on a motor-driven treadmill and continuous gas analyses of expired air as well as determinations of electrocardiographic changes in rate and pattern, blood pressure and arterial oxygen saturation (oximeter) (Figure 1). Observations have not been limited to the basal state in order to obtain an appraisal of performance to a standardized stress of exercise in relation to ordinary circumstances in regard to diet, clothing and activity. In a separate report the statistical analyses of variability of normal performances under these conditions is presented (4). For the purpose of defining the normal pathways in this report, only the mean values of all pertinent observations obtained at minute intervals before, during, and after exercise are graphically considered in relation to each other.

METHODS

Thirty-five normal adults were used for this investigation (20 men, 15 women, ranging in age from 20 to 57 years). Each was allegedly healthy and gainfully employed, and none had any detectable sequelae of any previous cardiac or pulmonary disease. All rested before

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EQUIPMENT FOR EXERCISE TOLERANCE TESTING

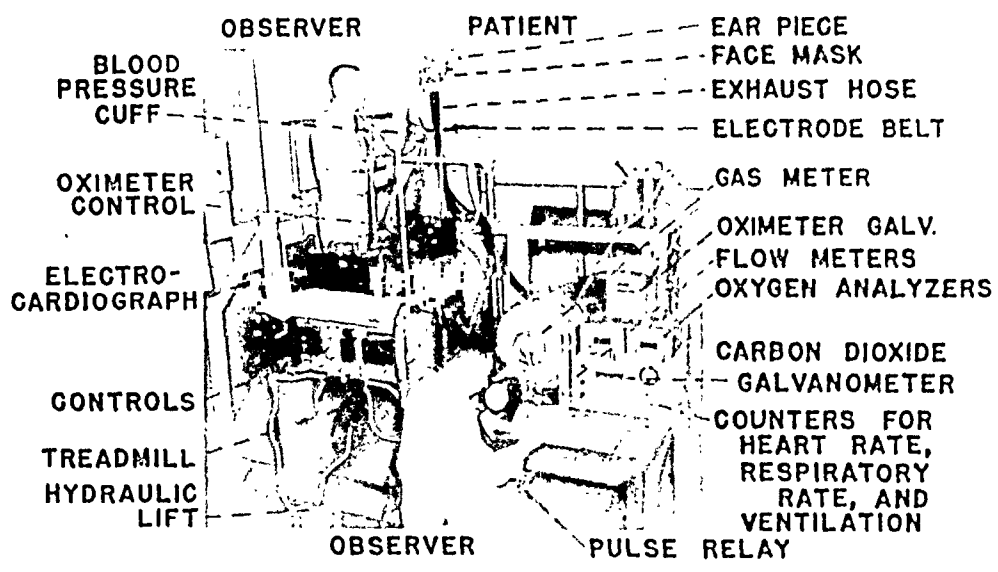


FIG. 1

Arrangement of equipment for exercise tolerance testing. One observer remains on the treadmill platform with the patient, records blood pressure, electrocardiogram, symptoms and signs. The other observer seated at the continuous analyzer records the heart rate, respiratory rate, ventilation volume, gas concentrations, and oximeter at one minute intervals.

the test period and then were observed during 10 minutes of rest sitting in a chair, 10 minutes level walking on the treadmill at the rate of 2.6 mph (12 rpm of the conveyor belt or 70 meters per minute velocity), and again for 10 minutes of recovery while sitting in a chair. Each was lightly clothed, and the barometric pressure and temperature were the prevailing room air values (747.3 ± 1.1 mm. Hg, 0.15% coefficient of variability) and $24.9 \pm 1.9^\circ$ C (7.9% coefficient of variability).

The systemic blood pressure was obtained from an aneroid sphygmomanometer at frequent intervals, even during walking. Changes in arterial oxygen saturation, as well as variations in characteristics of the ear (green filter), were followed by an improved Millikan oximeter. Three electrodes attached to a rubber belt around the subject's chest for "ground," "indifferent" (right scapula) and "apical" contacts permitted registration of the chest lead electrocardiogram on a direct-writing instrument as well as continuous recording of the pulse from the amplified QRS spikes. Expired air gas data were obtained from a modified A-13A oxygen mask through a $1\frac{1}{2}$ inch corrugated rubber hose mixing chamber conducting to a continuous gas analyzer. Ventilation volumes were electrically recorded from a low resistance (differential pressure less than 1 inch of water) wet-test gasometer with accuracy of $\pm 1.65\%$ at 100 liter per minute velocities. The respiratory rate was recorded by activation of a variable time-delay relay counter. Gas analyses were made continuously on aliquot (saturated with water) samples (at flow rates of 100-125 ml per minute regulated by calibrated rotameters),

by means of a thermal-conductivity cell for carbon-dioxide expressed in volumes per cent; and a suitable Pauling oxygen analyzer which determined the partial pressure of oxygen. The calibration of each was established and checked against room air prior to each test. The terminal portion of each expired breath (mid-capacity air)⁵ was continuously sampled by the alternate pulsations of a very thin rubber balloon incorporated in the face piece of the mask for analyses of oxygen tension by a second Pauling analyzer and the derivation of changes in the physiological dead space. These values included the dead space of the mask which was reduced by careful packing with cotton. At the rates of flow stated above, the aliquot gas samples reached the analyzers after a lag of 10-20 seconds for expired air and 40-50 seconds for the mid-capacity air. Since the response of the CO₂ analyzer was delayed to 99% peak reading in five minutes from zero concentration, acute changes in CO₂ concentration could not be followed with accuracy. Hence no correction for inspired air volume from differences in nitrogen concentration in expired air were made, and estimates of the expired air respiratory quotients were only approximated values (averaging about 4% too high), (Figure 2).

⁵ In the traditional sense, alveolar air has the composition of that volume of air which is obtained at the end of forced expiration, hence includes in the last portion of the reserve air partition of the pulmonary capacity. "Mid-capacity" air corresponds in gaseous composition to that obtained at the end of normal expiration.

MAGNITUDE OF ERROR IN OXYGEN CONSUMPTION

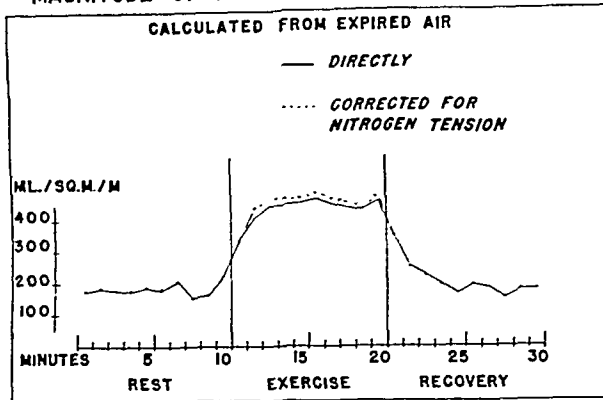


FIG. 2

The differences in oxygen consumption calculated directly are compared with those obtained by correction for changes in nitrogen tension. The magnitude of error by the short cut calculation is considered almost negligible in relation to the change in oxygen consumption initiated by exercise.

The reproducibility and accuracy of gas analyses by this method was estimated as *variations* of duplicate analyses from an ideal value of zero in terms of *standard deviations* as follows:

	CO ₂ vol. %	O ₂ vol. %
a) Haldane technique	± 0.054	± 0.058
b) Continuous analyzer	± 0.160	± 0.028
c) Between "a" and "b"	± 0.360	± 0.083 ^a

Consequently reliance was placed upon the oxygen values. Suitable conversion tables permitted rapid determination of the volumes per cent of oxygen absorbed (respiratory efficiency) from air from the observed expired air pO₂ reading and the dry barometric pressure at the operating temperature. Similarly a factor was established for each subject that permitted conversion of the ventilation volumes observed into liters per square meter of body surface area at 760 mm. Hg and 0° C dry. The oxygen consumption was estimated from the product of the corrected minute ventilation and the respiratory efficiency and ranged between 96-101% (depending on the R.Q.) of the true value based upon corrections for nitrogen differences in inspired and expired air. All primary observations were recorded and graphed at one minute intervals (Figure 3). From these data, average values per period, dead space volumes, oxygen debt, ventilation indices, etc. were calculated.

Accessory procedures routinely employed were the determination of maximum *mask* breathing capacity by means of voluntary hyperventilation for 30 seconds at the completion of the above test, changes in oximeter

^a The standard error for oxygen analyses determined by the Haldane technique was 3.3 times greater than that obtained by the Pauling oxygen analyzer.

readings with breath-holding, and estimation of lung-to-ear circulation time with the oximeter.

RESULTS

Inasmuch as *statistical analyses* showed no significant differences in the data according to the sex of the subjects, all the consecutive one minute observations for the mean values of both sexes have been compiled together. The resting values (allowing for differences in basal state, tempera-

EXERCISE PERFORMANCE (NORMAL MALE)

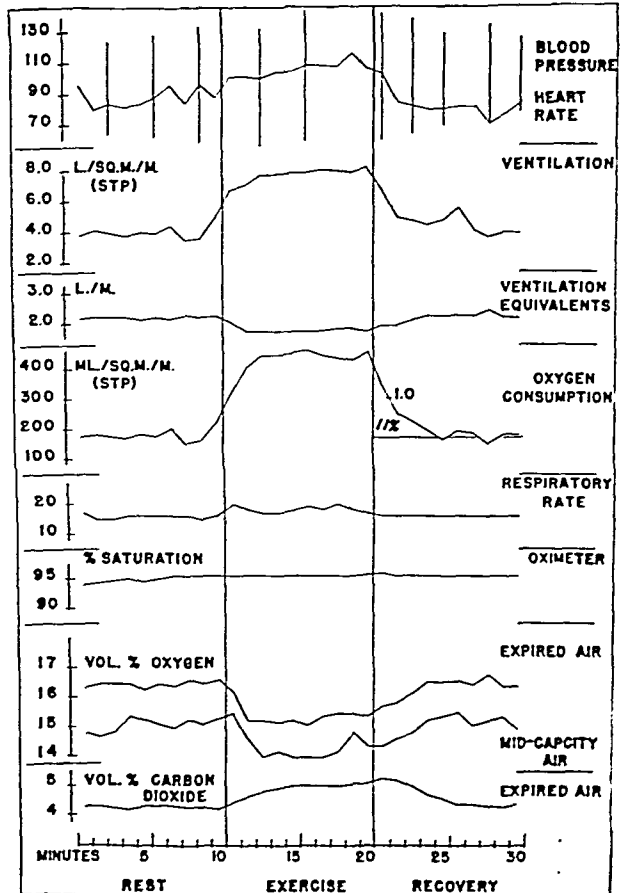


FIG. 3

Typical exercise performance graph of a normal male subject. The values of "11%" and "1.0" adjacent to the recovery curve for oxygen consumption refer to oxygen debt and half-time recovery time, respectively. The value of minute-by-minute observations of multiple measurements is illustrated by the fleeting changes in heart rate, ventilation, and oxygen consumption occurring on the seventh, 19th, and 28th minutes. These variations were involuntary reactions to environmental stimuli initiated by visitors during the period of observation. The subject was unaware of these variations.

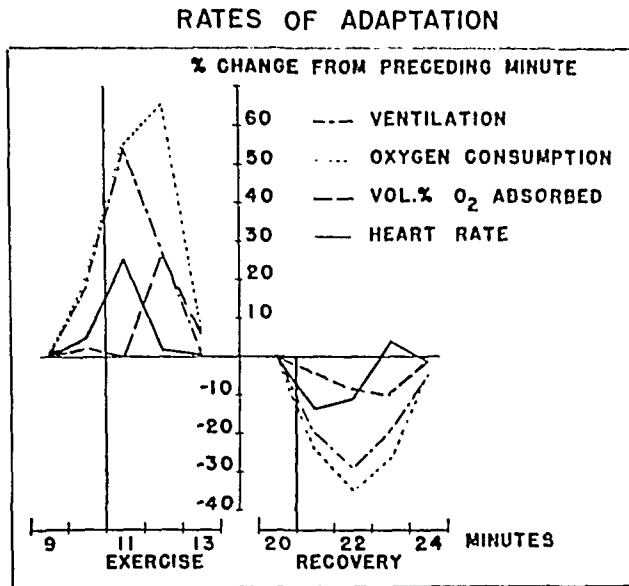


FIG. 4

The rates of adaptation to changing states of activity are compared on the basis of per cent change from the preceding mean one minute values for ventilation volume, oxygen consumption, volumes per cent of oxygen absorbed from expired air and heart rate for the first four minutes of exercise and recovery. The 10th minute represents changes from standing. Note that ventilation and oxygen consumption show the greater changes first, and that the change in volumes per cent of oxygen absorbed is delayed, causing the final increment in oxygen consumption during exercise.

ture and pressure) are in accord with those reported by others.

The *rates of changes* in the observations of ventilation, oxygen consumption, respiratory efficiency (volumes per cent of oxygen absorbed) and the heart rate are shown in Figure 4. These changes are expressed as percentage changes in the several factors from the immediately preceding minute during the periods of adaptation to and from a steady state of exercise. The values for the 10th minute of observation represent the changes induced by standing up and are of smaller magnitude than those for walking. As a result of exercise both ventilation and heart rate promptly rise, but the greatest change is confined to the first minute. The oxygen consumption initially rises in proportion to the ventilatory response, but thereafter it continues to rise during the next minute because of the greater respiratory efficiency. By the 13th minute of observation all factors have approached a new and relatively stable range during the steady state of exercise. In recovery the reverse changes promptly occur, but in this

instance there are two apparent differences: *a*) there is no lag in decline of respiratory efficiency, and *b*) the heart rate is restored toward the resting level more rapidly than the other factors.

There are changes in the physiological *dead space* along with the increments in the *mid-capacity ventilation* associated with exercise (Figure 5). The latter shows an almost linear rise during the first three minutes of exercise as well as a linear decline in three minutes during recovery from exercise. During the last seven minutes of exercise there is a just perceptible increase in the mid-capacity ventilation. The slight discrepancies between total ventilation and mid-capacity ventilation appear to be related to fluctuations in the physiological dead space. On the average, throughout the three periods of observation the dead space represents 21% of the tidal volume. Both the relative and absolute values for the dead space are lower than those reported by others (about 26 to 30% [5, 6]), and recent improvements in the face mask sampling device yield somewhat higher dead space values indicating a technical error as partly responsible for these discrepancies. Although the dead space shows a

MEAN INTRAPULMONARY VENTILATION

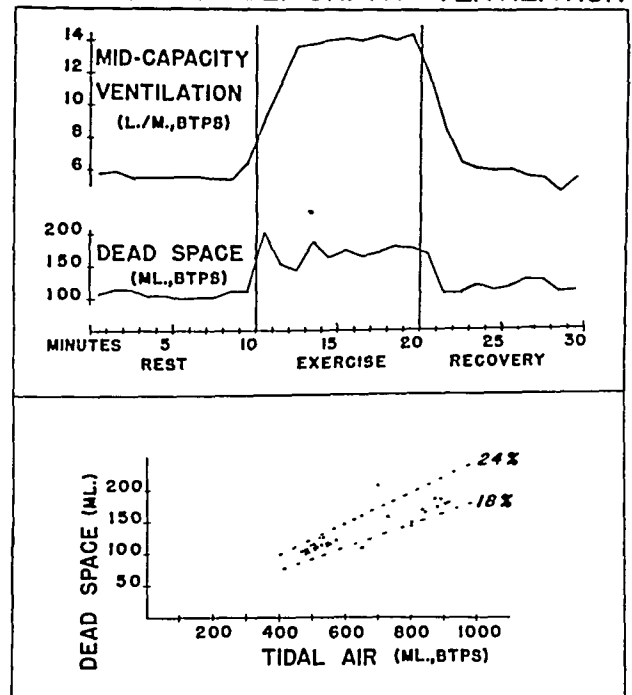


FIG. 5

Relationships of mean intrapulmonary ventilation, physiological dead space, and tidal air volume in ambient values, B. T. P. S., in normal subjects.

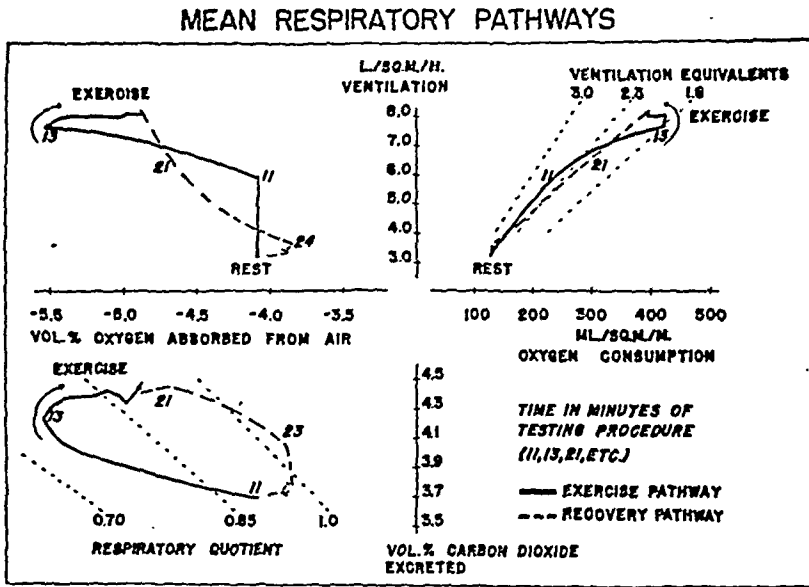


FIG. 6

The mean respiratory pathways in normal subjects illustrate the various inter-relations between ventilation, oxygen consumption, ventilation equivalents for oxygen, respiratory quotient, and changes in activity from rest to exercise and back to rest again. The arrows indicate the direction of the loops, and the numbers adjacent to the loops indicate the specific minutes of observation during the testing procedure. The iso-R.Q. lines are indicated by the background grid; note that the ultimate exercise R.Q. is slightly lower than the resting value. (Inasmuch as the oxygen consumption and dead space change with activity, iso-ventilation lines cannot be applied to this graph). Note that the ventilation equivalent for oxygen is lower during exercise than at rest, indicating increased pulmonary capillary flow and more effective oxygenation of the pulmonary arterial blood. See text for further discussion.

disproportionately large value early in exercise followed by a transient decline, the average dead space value during exercise is 62% larger than the resting values.

The mean respiratory pathways of adaptation from a steady state of rest to that of exercise and the converse during recovery are shown in Figure 6. The oxygen consumption at first increases proportionately with the increments in ventilation, but a little later it rises more rapidly because of greater respiratory efficiency (volumes per cent of oxygen absorbed, or reciprocally, diminishing ventilation equivalents for oxygen). The improved respiratory efficiency is a function of more effective alveolar ventilation and circulation as well as alterations in blood flow and composition (see below). Concurrently the physiological dead space enlarges. After the 13th minute of observation (third minute of exercise), the estimated

oxygen consumption gradually declines 5% as the respiratory efficiency slowly recedes (Figure 6) with a perceptible rise in mid-capacity ventilation (Figure 5). On the average there is a 22% increase in respiratory efficiency at this level of work. The estimated R.Q. of expired air shows wide fluctuations during the adaptive phases because of the differences in rates of change in the several factors representing ventilation and circulation. The direction of change in the R.Q. is always a clockwise loop showing a low value early in exercise as the highest values for respiratory efficiency are achieved by the 13th minute of observation (Figure 6). During steady states of performance, either rest or activity, the expired air R.Q. may be considered representative of tissue metabolism throughout the body. With this level of work, the R.Q. does not rise above the resting value as it does with more severe levels of work

MEAN CIRCULATORY PATHWAYS

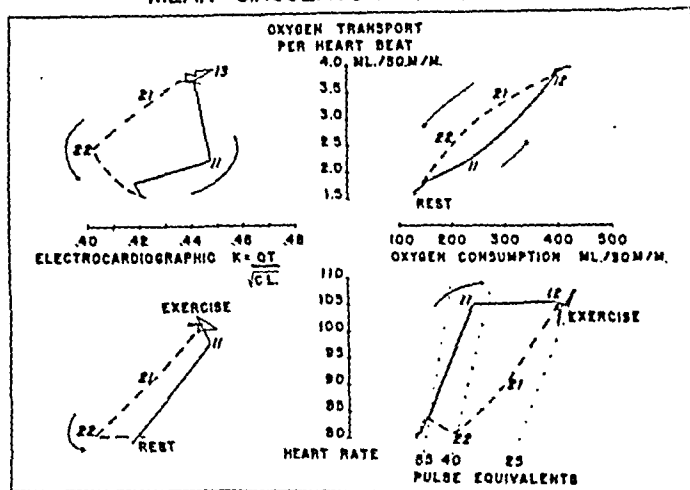


FIG. 7

The mean circulatory pathways in normal subjects illustrate the various inter-relations of heart rate, oxygen consumption, pulse equivalents for oxygen, amount of oxygen carried per heart beat and electrocardiographic K constant during changes in activity from rest to exercise, and back to rest again. Note that the heart rate rises rapidly during the first minute, and maintains a plateau thereafter during exercise. The decline in pulse equivalents indicates a rise in stroke volume. The arrows indicate the direction of the loops, and the numbers adjacent to the loops indicate the specific minutes of observation during the testing procedure.

(7). With the onset of recovery the $R.Q.$ of expired air transiently exceeds 1.0 as retained CO_2 continues to be excreted in excess of the rate of oxygen absorption reduced with diminished tissue demands for oxygen.⁷ Simultaneously the level of ventilation is maintained above the tissue demands by the stimulus of retained CO_2 , and concurrently most of the aerobic oxygen debt is repaid.

The *mean circulatory pathways*, based upon consecutive observations of minute heart rate, oxygen consumption, oxygen transport per heart beat, and systolic time are shown in Figure 7. The systolic time is derived from the (chest lead)

⁷ Pelnar utilizes the abnormally high $R.Q.$ values during steady states of either rest or moderate activity to recognize the presence of hyperventilation, beyond the metabolic needs of the tissues, caused by neuropsychogenic factors. The usage of the term "respiratory quotient" in this sense may be unfortunate, especially when consideration is given to the limitations in accurately measuring the concentration of carbon dioxide by the present authors. Possibly the term "respiratory ratio" or ratio of carbon dioxide excreted to oxygen absorbed would be more appropriate.

electrocardiographic changes in the K constant, or observed QT interval divided by the square root of the cycle length to correct for changes due to rate. The oxygen transport is derived from the following equations:

(a) Cardiac Output

$$= \text{Heart rate} \times \text{stroke volume} \\ = \frac{\text{Oxygen consumption}}{\text{A-V } O_2 \text{ difference}}$$

and by substitution:

(b) Oxygen Transport

$$= \frac{\text{Oxygen consumption}}{\text{Heart rate}} \\ = \text{Stroke volume} \times \text{A-V } O_2 \text{ difference.}$$

The oxygen transport also represents the average rate of oxygen transfer from either alveolar air to mixed venous blood, or arterial blood to peripheral tissues, per unit of physiological time of one heart beat. Statistical analyses of rates of change in large series of observations on normal subjects show that there is a 7% increase in cardiac output for every 10% increment in oxygen consumption, and that the stroke volume shows somewhat greater increases than the A-V oxygen difference with increasing cardiac output (8). Similarly analyses of the present series of mean values shows a linear relationship of a high order of significance between oxygen consumption and oxygen transport ($r = 0.938$). With the initiation of exercise the heart rate promptly accelerates during the first minute of exercise and thereafter remains on a plateau (Figure 4). Since the oxygen consumption continues to rise, and hence the cardiac output, further increases in cardiac output must be achieved by larger stroke volumes. The rates of change in oxygen transport show the greatest increase during the second minute of exercise when the A-V oxygen difference probably is also widening due to a reduction of mixed venous oxygen content caused by blood returning from the exercising muscles (7). Concurrently the respiratory efficiency is attaining its highest and the $R.Q.$ its lowest values. During these adaptive changes in circulation (Figure 3) there are corresponding changes in the systolic time manifested by the electrocardiographic K constant. Initially with exercise there is an appreciable rise during the first minute followed by gradual fluctuations dur-

ing the remainder of exercise; on the average the exercise systolic time is 7% greater than that observed during the resting period. With recovery the K value falls during the 21st and 22nd minute of observation to 9% below the exercise range and then gradually resumes the resting value. In none of the normal subjects studied at this level of work were there any significant alterations of the QRS complexes, ST segments, or T waves, other than the effects of a more rapid rate; *i.e.*, no changes in the electrocardiogram that could be associated with possible or probable insufficiency of the coronary circulation.

DISCUSSION

The continuous recording of the heart rate electrocardiographically, ventilation volume, and respiratory efficiency of expired and mid-capacity air, as well as the carbon dioxide concentration during changes in the metabolic demand initiated by exercise greatly supplements the existing information about either ventilatory performance or alterations in gaseous composition. By these means the pathways of respiratory and circulatory adaptation to the stress of exercise can be observed in terms of degrees, as well as rates, of change in a manner that demonstrates the complementary sparing action of the several variables. Appreciable uniformity of results can be approximated by control of the work-load in proportion to the body size by means of walking on a motor-driven treadmill. These studies have not been performed under basal conditions in order to observe the responses in both normal subjects and patients with cardio-respiratory diseases under ordinary circumstances of daily activity. An analysis of the range of variability and reproducibility of results in normals and alterations presented by patients with disease will be the subject of later reports. The resting values observed by these methods are in reasonable accord with those observed by others employing the Tissot-Haldane methods (9). The pathways of responses for expired air are similar to the findings of Rahn and Otis (1) for normal alveolar pathways in exercise as well as those of Pelnar (2) for the changes in expired air composition initiated by exercise.

These studies demonstrated the following changes which regularly occur with exercise:

ventilation and heart rate promptly rise to a higher plateau, but the heart rate levels off by the second minute of exercise whereas the mid-capacity ventilation continues to increase in a linear fashion for three minutes. The R.Q. of expired air initially declines with exercise as the respiratory efficiency (denominator, or volumes per cent of oxygen absorbed) rises and overshoots the steady state value by the third minute of exercise (Figure 6). This transient change is an expression of greater alveolar ventilation and circulation (together with enlarged physiological dead space) as well as greater diffusion of oxygen into more rapidly circulating blood which has a lower content of oxygen as a result of increased muscular demand in exercise (7). The arterial oxygen saturation is maintained; hence the A-V oxygen difference widens; and since the oxygen consumption continues to rise for three minutes (and heart rate does not), there is a progressive increase in stroke volume. The striking increase in oxygen transport per heart beat during the second minute of exercise is an expression of the changes in both the A-V oxygen difference and the stroke volume since it is the product of these two factors. As the apparent circulatory lag in adaptation to exercise is corrected, the expired air R.Q. gradually rises during the further attainment of the steady state of exercise and the respiratory efficiency slowly declines; ventilation just perceptibly rises; and oxygen consumption diminishes by about 6%. These changes are considered to reflect peripheral circulatory adjustments as vasodilatation occurs to dissipate heat and slight arteriolization of venous blood from small peripheral shunts permits the mixed venous oxygen content to rise (and narrow the A-V oxygen difference) (10, 11). With these changes there is no significant change in diastolic blood pressure although the systolic pressure, and hence, the pulse pressure increase. The alterations in the systolic time expressed by the electrocardiographic K constant are considered expressions of changes in blood flow as well as contractile force and ventricular emptying associated with increased stroke volume. Some individuals exhibit slight reductions in both heart rate and ventilation about the seventh minute of exercise, indicating further adjustments in performance; possibly these changes account for the phenomenon of the "second wind"

subjectively observed at more severe levels of work.

Recovery from exercise begins at once, and in normal subjects at this level of work it is largely completed within three minutes. The first variable to approach the resting level is the heart rate which corroborates the usefulness of the recovery pulse as a measure of physical fitness (12). During this time the expired air R.Q. rises above 1.0 as retained carbon dioxide continues to be excreted, delaying somewhat the restoration of ventilation to the resting level although favoring the repayment of most of the aerobic oxygen debt.

SUMMARY

1. The mean respiratory and circulatory pathways of adaptation to the stress of exercise have been studied in 35 normal adults. The work-load was regulated by treadmill walking, and multiple observations were made continuously at one minute intervals before, during, and after the exercise.

2. The previously reported changes in gas composition were confirmed.

3. The additional values of simultaneous consideration of circulatory changes are discussed.

4. The adaptive changes in respiration and circulation are shown to complement and spare each other by the differences in time and rate of change.

ACKNOWLEDGMENT

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VARIABILITY OF RESPIRATORY AND CIRCULATORY PERFORMANCE DURING STANDARDIZED EXERCISE¹

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In the preceding report (1) the complementary adaptations of respiration and circulation to the stress of exercise in normal subjects were described. Various isolated measurements, obtained by a method of continuous observation, were found to be in accord with those reported by earlier investigators employing the established Tissot-Haldane techniques of respiratory gas analysis. Because of the continuity of observation, various rates of change in adapting from a state of rest to a standardized stress of exercise (treadmill walking) as well as rates of recovery could be observed in relation to each other as loops of cardio-respiratory responses. These respiratory and circulatory pathways portray the representative responses in normal subjects, but a survey of the *range of variability* in normals and patients is needed before these pathways can be utilized properly for the study of impaired functions in patients with cardio-respiratory diseases. Pelnar (2) has critically reviewed the existing methods of functional appraisal of the causes of dyspnea in patients and expressed the opinion that his new method based upon the continuous study of the R.Q. curve during rest, exercise and recovery provides a more satisfactory appraisal of function in relation to dyspnea than any other method previously available. If these observations can be confirmed, then continuous observation of standardized exercise may provide the means of rating disability in relation to functional impairment of respiration and circulation and possibly differentiating between the two chief types of dyspnea in patients: pulmonary insufficiency and circulatory insufficiency.

It is the purpose of this paper to analyze statistically the data obtained from the same 35 normal

adults previously reported (1) to determine the range of normal variation for the several measurements. By this means the minimum and maximum value for each factor during rest, exercise or recovery can be determined. Secondly, the magnitudes of change in the several factors in normal subjects performing at this standardized workload are assessed in both normal subjects and patients with various diseases causing clinical symptoms and signs of functional impairment. Hence the necessary percentage change in the various exercise measurements for a significant difference in the same individual on repeated examinations was estimated. Thus the comparative value of the various measurements made can be estimated to ascertain those which are more reliable for discriminating functional impairment and rating physiological disability.

METHODS

Statistical analyses consisted of determination of the standard deviation, standard error and coefficient of variation for each of the observations in the same 20 normal males and 15 normal females previously reported in terms of mean values. The standard deviation of the mean values was calculated according to the formula

$$\sigma_m = \sqrt{\frac{\sum d^2}{N-1}},$$
 assuming, of course, that our sample

was truly a representative group. Standard error was calculated according to the formula $SE_m = \frac{\sigma_m}{\sqrt{N-1}}$,

and the coefficient of variability was calculated as $\frac{\sigma_m}{\text{mean}}$ multiplied by 100 to express all values in per cent. Because two standard deviations above and below the mean include approximately 95% of the range that can be expected on the basis of random sampling, the limits of normality are tabulated as plus or minus two standard deviations from the mean values for the periods of rest, exercise and recovery.

⁴ σ_m = standard deviation of mean; d = difference of observation from the mean; Σ = summation; N = degrees of freedom.

¹ Aided by grants from the Hochstetter Fund.

² Bertha Hochstetter Buswell Research Fellow in Medicine.

³ Fellow of the General Education Board.

TABLE I
Statistical analysis of all measurements (20 males—15 females)

Factors analyzed	Units	Mean		Standard deviation		Standard error		Coefficient of variability (per cent)	
		M	F	M	F	M	F	M	F
Age	years	30.2	32.2	5.3	10.2	1.21	2.73	17.5	31.7
Arterial oxygen saturation (oximeter)	per cent								
Rest		95	96	1	3	.24	.83	1.1	3.1
Exercise		95	97	2	4	.48	1.11	2.1	4.5
Recovery		95	98	2	4	.48	1.11	2.1	4.5
Breath-holding time	seconds	37	38	17	8.3	4.8	2.3	46.0	21.8
Cardiovascular data									
Circulation time (lungs to ear)	seconds	5.6	5.2	1.	1.1	.23	.30	17.8	21.2
Heart rate									
Rest	beats min.	80	81	11.0	7.7	2.67	2.06	13.7	9.5
Exercise		102	107	11.0	3.0	2.67	2.43	10.8	8.5
Recovery		82	85	12.0	7.9	2.91	2.11	14.6	9.3
Systolic pressure									
Rest	mm. Hg	124	118	10.0	11.0	2.36	2.94	8.1	9.3
Exercise		139	136	14.0	18.0	3.30	4.82	10.1	13.2
Recovery		128	121	10.0	13.0	2.36	3.42	7.8	14.9
Diastolic pressure									
Rest	mm. Hg	80	78	11.0	6.0	2.6	1.60	13.7	7.7
Exercise		82	78	10.0	7.0	2.36	1.87	12.2	9.0
Recovery		82	79	9.0	7.0	2.12	1.87	11.0	8.8
Pulse pressure									
Rest	mm. Hg	44	40	10.0	8.0	2.36	2.14	22.7	20.0
Exercise		55	58	13.0	16.0	3.07	4.28	23.6	27.6
Recovery		46	42	7.0	11.0	1.65	2.94	15.2	26.2
Metabolic demands									
Calories									
Rest	cal. min.	1.28	1.02	.22	.15	.051	.041	17.5	15.0
Exercise*		2.56	2.26	.41	.66	.95	1.71	16.1	29.2
Ratio of $\frac{\text{Exercise cal.}}{\text{Resting cal.}}$		2.02	2.22	.11	.16	.025	.044	5.4	7.5
Oxygen consumption									
Rest	ml./sq.m./min. STPD	137	125	21.0	16.0	4.8	4.3	15.3	12.8
Exercise		393	379	47.0	55.0	10.8	14.7	11.9	14.5
Recovery		160	153	22.0	14.0	5.1	3.7	13.8	9.1
Oxygen debt	per cent	8	10	3.0	3.0	.69	.81	37.5	30.0
Half-time recovery	min.	.8	1.1	0.2	0.3	.046	.08	25.0	27.2
Oxygen transport									
Rest	ml./sq.m./min. STPD	1.72	1.54	.26	.19	.059	.058	15.1	12.3
Exercise		3.83	3.44	.24	.28	.055	.075	6.3	8.1
Recovery		1.96	1.82	.32	.21	.074	.056	16.3	11.5
Respiratory gases									
Oxygen absorbed (expired air)									
Rest	vol. %	4.18	3.86	.39	.34	.089	.091	9.3	8.8
Exercise		5.17	4.86	.39	.45	.089	.12	7.5	9.3
Recovery		4.14	3.86	.39	.34	.089	.09	9.4	8.8
Carbon dioxide excreted (expired air)									
Rest	vol. %	3.91	3.43	.50	.44	.11	.12	12.8	12.8
Exercise		4.64	4.14	.53	.44	.12	.12	11.4	10.6
Recovery		4.23	3.82	.55	.44	.13	.12	13.0	11.5
Respiratory quotient (expired air)									
Rest	ratio	.94	.88	.03	.08	.007	.021	3.4	9.4
Exercise		.89	.85	.03	.08	.007	.021	3.4	9.4
Recovery		1.02	.99	.04	.09	.009	.024	3.8	9.1
Oxygen absorbed from mid-capacity air									
Rest	vol. %	5.33	4.89	.48	.37	.11	.09	9.0	7.6
Exercise		6.41	6.01	.28	.48	.066	.128	4.4	8.0
Recovery		5.39	4.99	.30	.36	.071	.096	5.5	7.2

TABLE I—Continued

Factors analyzed	Units	Mean		Standard deviation		Standard error		Coefficient of variability (per cent)	
		M	F	M	F	M	F	M	F
Surface area	sq.m.	1.88	1.66	.13	.14	.029	.037	6.9	8.4
Ventilation	(STPD)								
Maximum mask breathing capacity	L/sq.m./min.	30.3	29.3	6.0	5.2	1.4	1.4	19.8	17.7
Minute ventilation									
Rest	L/sq.m./min.	3.28	3.25	.54	.50	.124	.134	16.5	15.3
Exercise		7.57	7.86	.93	1.19	.213	.318	12.3	15.1
Recovery		3.84	3.91	.55	.49	.126	.131	14.3	12.5
Respiratory rate									
Rest	breaths/min.	14	15	2.76	2.6	.63	.69	19.7	17.3
Exercise		18	22	3.03	3.5	.69	.93	16.8	15.9
Recovery		14	16	3.21	2.5	.73	.67	22.9	15.6
Tidal air									
Rest	ml./sq.m./min.	252	226	67	38	15.	10.	26.5	17.0
Exercise		434	367	81	66	18.6	17.6	18.7	18.0
Recovery		285	251	72	33	16.5	8.8	25.2	13.1
Ventilation equivalents for oxygen	L/min.								
Rest		2.42	2.63	.22	.24	.05	.06	9.1	9.1
Exercise		1.96	2.11	.12	.21	.027	.056	6.1	9.9
Recovery		2.46	2.66	.24	.24	.055	.064	9.7	9.0
Ventilation index	ratio	5.4	7.1	1.0	2.3	.23	.61	18.5	32.0
Vital capacity (STPD)	L/sq.m.	2.1	1.8	.3	.4	.068	.107	14.3	22.0
Average vital rate	L/sec.	1.7	1.4	.5	.7	.11	.20	29.4	50.0

* Corrected for R.Q. and Oxygen Debt

Variations within each of the three periods of observation were studied similarly for the basic primary measurements of heart rate, ventilation volume, and respiratory efficiency (volumes per cent oxygen absorbed). Each item was measured at one minute intervals consecutively, and the standard deviation of the mean values was calculated for all 35 normal subjects. By this method, one thus obtains a measure of the variability of minute-to-minute analysis in any one period.

The magnitude of change from one period to another in the various factors was expressed as percentage change from the resting values during exercise and recovery.

The reproducibility of exercise performance was determined by analysis of the standard deviation of the mean value for repeated tests on the same individual. This analysis also includes seven abnormal patients upon whom repeated tests were made. Coefficients of variability were determined in this group as described above for the average heart rate, ventilation volume and respiratory efficiency, oxygen consumption, volumes per cent of carbon dioxide excreted in expired air, estimated R.Q., duration of walking in minutes, oxygen transport per heart beat and oxygen debt. All gas volumes were expressed in terms of STPD, corrected for surface area. The patients utilized for this purpose had the following clinical diagnoses: chronic pulmonary granulomatosis associated with beryllium compounds (two), bronchiostenosis following organizing pneumococcal pneumonia (one), pericardiectomy for relief of constrictive pericarditis (one), coronary insufficiency following healed coronary

occlusions (two), and congenital heart disease due to pulmonary stenosis and auricular septal defect (one). The period of time for these observations ranged from one month to 12 months.

RESULTS

1. Statistical variations between normal subjects to the standardized exercise test (2.6 mph level walking for 10 minutes)

The observations on 35 normal adults are summarized in Table I in terms of mean values, standard deviations, standard errors, and coefficients of variability according to sex. There is no significant difference⁵ between sexes,⁶ and accordingly the averages for men and women for the several

⁵ A difference is considered significant when there is less than one possibility in 369 of being due to chance selection, or there is a three-fold difference between the standard errors.

⁶ The apparent difference in resting oxygen saturation is an artefact since the calibration of the oximeter is arbitrary. The ventilation index is almost significantly higher in men than women, largely because the vital capacity (used in the denominator of the formula) is slightly smaller in women. Ventilation index equals the sum of the average exercise and recovery minute ventilation values divided by the vital capacity, all expressed in volumes corrected for STPD and body surface area.

TABLE II
Normal limits plus or minus two standard deviations from combined mean value (male and female)

		Mean	Min.	Max.
Breath-holding	seconds	37.5	12	63
Circulation time	seconds	5.4	3.2	7.6
Heart rate				
Rest	beats/min.	81	62	99
Exercise		104	90	118
Recovery		84	61	107
Systolic pressure				
Rest	mm. Hg	121	101	142
Exercise		138	106	170
Recovery		125	102	148
Diastolic pressure				
Rest	mm. Hg	79	62	96
Exercise		80	63	97
Recovery		81	65	97
Pulse pressure				
Rest	mm. Hg	42	24	60
Exercise		56	27	85
Recovery		44	26	62
Calories				
Rest	cal./min.	1.16	.79	1.53
Exercise		2.43	1.36	3.50
Ratio $\frac{\text{Exercise}}{\text{Rest}}$		2.09	1.82	2.36
Oxygen consumption				
Rest	ml./sq.m./min.	132	95	169
Exercise	STPD	381	279	483
Recovery		156	120	192
Oxygen debt	per cent	9	3	15
Half-time recovery of oxygen consumption	min.	.94	0.5	1.4
Oxygen transport				
Rest	ml./sq.m./heart beat	1.64	1.16	2.12
Exercise	STPD	3.65	3.13	4.17
Recovery		1.89	1.35	2.43
Respiratory efficiency (expired air)	vol. %			
Rest		4.04	3.31	4.77
Exercise		5.03	4.19	5.87
Recovery		4.02	3.29	4.75
Carbon dioxide excreted (expired air)	vol. %			
Rest		3.70	2.76	4.64
Exercise		4.42	3.45	5.39
Recovery		4.05	3.06	5.04
R. Q. (respiratory ratio)				
Rest		.91	.80	1.02
Exercise		.87	.76	.98
Recovery		1.01	.88	1.14
Mid-capacity respiratory efficiency	vol. %			
Rest		5.14	4.29	5.99
Exercise		6.23	5.47	6.99
Recovery		5.21	4.55	5.87
Ventilation:				
Maximum mask breathing capacity	L/sq.m./min.	29.8	18.6	41.0
Minute Ventilation	STPD			
Rest		3.27	2.23	4.31
Exercise		7.69	5.57	9.81
Recovery		3.87	2.83	4.91
Respiratory rate				
Rest	breaths/min.	14	9	19
Exercise		20	14	26
Recovery		15	9	21
Tidal air				
Rest	ml./sq.m./min.	240	135	345
Exercise		405	258	552
Recovery		270	165	375
Ventilation equivalents for oxygen				
Rest	L/min.	2.51	2.05	2.97
Exercise		2.02	1.69	2.35
Recovery		2.56	2.06	3.04
Vital capacity	L/sq.m.	1.97	1.27	2.67

measurements are combined for purposes of all subsequent discussion. Allowing for differences in temperature and basal state of activity, the observed mean values for resting ventilation and oxygen consumption show satisfactory agreement with those reported by others using the Tissot-Haldane method of gas analysis (3). The volume per cent of oxygen absorbed (or ml. per liter of air) is in exact agreement with previously reported values (2, 3). The measurements with coefficients of variability of *less than 10%* are the estimations of the arterial oxygen saturation, respiratory quotients, volumes per cent of oxygen absorbed from either expired air or mid-capacity air, and the ventilation equivalents for oxygen. In contrast the heart rate, blood pressure, pulse pressure, ventilation volume, oxygen consumption, calories of heat production, ventilation index and vital capacity all exhibit greater variability. Many of these factors reflect changes due to emotional stimuli, and some may be slightly affected by differences in environmental temperature⁷ as well as the type and amount of clothing worn—factors which are not controlled in this study. The overall variability for all the measurements throughout the three periods of observation is about 12%.

The *consecutive one minute variations* in the heart rate, ventilation volume and expired air analysis for oxygen concentration, expressed in terms of standard deviations, are shown in Figure 1. The last of the three listed is the least variable. The standard deviations are of the same order of magnitude throughout the three periods of observation.

The *limits of normality* for the several factors studied are shown in Table II. These minimal and maximal values have been determined arbitrarily as plus or minus two standard deviations from the combined mean values for both males and females. All volume measurements are expressed at STPD values corrected for surface area per unit time of one minute. This is to facilitate estimation of the oxygen consumption by the short-cut method previously described. The respiratory gas concentrations are expressed in terms of volumes per cent to eliminate minor

differences related to daily fluctuations in barometric pressure which would be reflected in partial pressure values for oxygen. (Alveolar $p\text{CO}_2$ appears to be homeostatically regulated by ventilation and is less dependent upon barometric fluctuations.) The maximal blood pressure values are above the conventional range of 140/90 mm. Hg in part perhaps because of the erect posture, but more likely because of vasomotor responses to apprehension about the testing procedure. Since these individuals often show lower pressure values when relaxed in the recumbent position, a more liberal interpretation of significant blood pressure increments may be necessary. Similar comments apply to measurements of heart rate, ventilation volume and oxygen consumption—all of which reflect responses to anxiety. It is apparent from these considerations that much of the spread of normal variation is dependent upon psychoneurogenic stimuli which of course may operate in either normal individuals or those with functional impairment from cardio-respiratory diseases. Since there is less variation in concentrations of the respiratory gases, and rates of CO_2 excreted to O_2 absorbed per unit volume of ventilation may be a direct measure of neuropsychogenic hyperventilation, Pelnar's opinion that these measurements afford a more reliable appraisal of performance in patients with dyspnea is strengthened (2).

2. Magnitude of changes in normal subjects to the standardized exercise test

The amount of work done by this type of exercise is moderate since the average working caloric expenditures is only 2.1 times above the resting value.⁸ The magnitudes of changes caused by this amount of work are summarized in Table III as percentage differences from the corresponding resting measurements. Whereas the ventilatory reserve, respiratory quotient and ventilation equivalents for oxygen diminish somewhat during exercise, the remainder of the factors observed increase. The increments range from 1.5% for diastolic blood pressure to 193% for oxygen consumption. There is an average increase of 108% in calories of heat production, 135.5% in ventilation volume, 29.5% in heart rate, and 124.0% in oxygen

⁷ Statistical evaluation of the environment showed barometric pressure to be 747.3 ± 1.13 mm. Hg (0.15% coefficient of variability) and room temperature to be $24.9 \pm 1.9^\circ \text{C}$ (7.9% coefficient of variability).

⁸ Hard work is not performed until the metabolic rate is at least three times greater than the basal metabolic rate expressed in calories.

VARIATIONS FROM MEANS EXPRESSED AS STANDARD DEVIATIONS

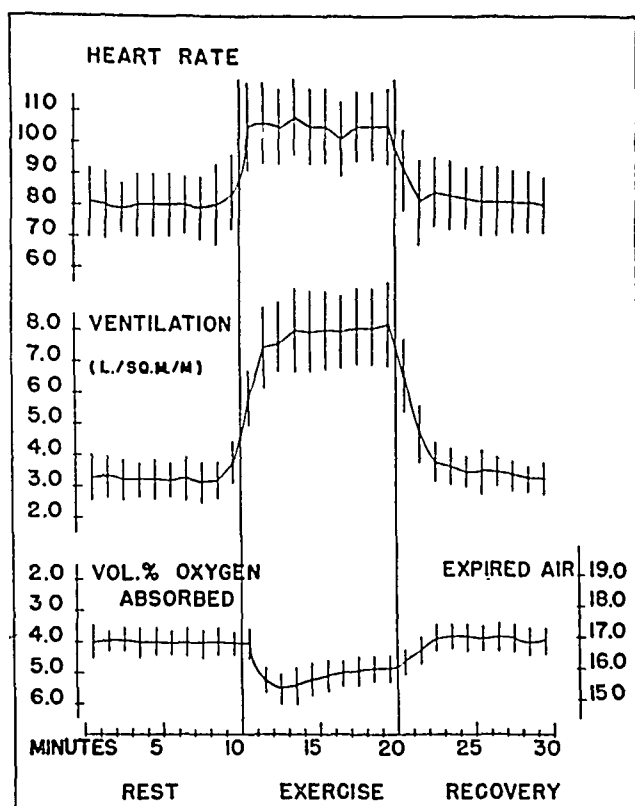


FIG. 1

Minute-by-minute mean heart rates, ventilation volumes, and volumes per cent of oxygen absorbed from expired air in normal subjects, together with range of variations expressed as standard deviations from each mean value. The vertical lines for each minute represent the variation of plus or minus one standard deviation from the corresponding mean. The expired air values on the right side of the graph are in terms of volumes per cent.

transport per heart beat, which is a measure of the change in the A-V oxygen difference and the stroke volume.⁹

3. Variability of multiple exercise tests in the same individuals

Table IV presents the statistical findings obtained from the study of 17 exercise tests in normals, and 54 tests in seven abnormal patients with clinical cardio-respiratory impairment. All tests were done at the rate of 2.6 mph level walking for 10 minutes, or to the limits of tolerance for those

⁹ Any variations in blood volume, hematocrit (4), or arterial oxygen saturations are considered insignificant in relation to the magnitude of change in stroke volume and A-V oxygen difference indicated by the change in oxygen transport per heart beat.

who could not walk for as long as 10 minutes, and the data are based upon the average of the one minute observations serially throughout exercise. It will be seen that in all instances the coefficient of variability for the averages of the normals is less than the corresponding value for the averages of the abnormals, except for oxygen debt where the reverse relationship is found. The factors which exhibit the least variability for either normals or abnormals are the duration of walking ("endurance"), respiratory efficiency, heart rate, oxygen consumption, oxygen transport per heart beat, and ventilation volume. The appreciably greater variability of values for volumes per cent of CO₂ excreted and respiratory quotient are attributed to technique, since the CO₂ analyzer employed is neither as stable nor accurate as the oxygen analyzer. Hence these observations cannot be interpreted to invalidate Pelnar's claim that observations of the R.Q. and CO₂ concentration are as important and reliable as those for oxygen concentration.

From these data the limits of variation observed by this standardized exercise test for both normals and abnormals can be estimated from the coefficient of

TABLE III

Exercise and recovery mean values in normal subjects (expressed as percentage change from resting measurements)

	Exercise	Recovery
<i>Decrease during exercise</i>		
Ventilatory reserve	-16.7	-2.1
Respiratory quotient	-5.3	+10.0
Ventilation equivalents for oxygen	-25.4	+1.2
<i>Increase during exercise</i>		
Cardiovascular measurements:		
Heart rate	29.5	3.7
Systolic pressure	13.5	2.8
Diastolic pressure	1.5	2.0
Pulse pressure	32.4	4.2
Oxygen transport per heart beat	124.0	15.9
Calories of heat production*	108.0	—
Oxygen consumption	193.0	18.9
Respiratory gases		
Vol. % O ₂ absorbed	24.8	-0.5
Vol. % CO ₂ excreted	19.4	9.2
Mid-capacity vol. % O ₂	22.0	1.5
Ventilation		
Total minute ventilation	135.5	18.3
Respiratory rate	37.5	2.8
Tidal air	69.1	12.0
Mid-capacity ventilation	141.0	29.0
Dead space	68.0	19.6

* Calculated from oxygen consumption, observed R. Q. and corrected for oxygen debt in recovery.

TABLE IV
Variability of multiple exercise tests (2.6 mph level walking, expressed as mean exercise values, standard deviations and coefficients of variation)

Diagnosis	Number of tests	Heart rate	Ventilation volume	Respiratory efficiency	Oxygen consumption	Vol. % CO ₂	R. Q.
Normal	4	91± 1.0	6.72± .18	4.85±.16	327± 7.6	3.96± .33	.85±.06
Normal	8	91± 4.4	9.06± .23	4.18±.19	378±14.0	3.80± .40	.87±.11
Normal	5	101± 6.0	7.04± .70	5.05±.20	357±37.0	4.28± .41	.82±.05
Average		94± 3.8	7.61± .37	4.70±.18	354±19.5	4.01± .38	.85±.07
		4.0%	4.9%	3.9%	5.5%	9.5%	8.6%
Beryl. granul.	15	143±10.7	12.56±1.27	3.03±.17	376±25.7	2.16± .09	.74±.03
Beryl. granul.	18	122± 5.8	12.61± .61	2.93±.10	360±23.9	2.38± .62	.83±.20
Bronchostenosis	5	94± 8.4	10.95± .42	3.20±.24	352±25.0	2.95± .40	.93±.10
Pericardiectomy	4	113± 7.2	8.40± .83	4.95±.40	413±10.0	3.90± .60	.79±.11
Coron. insuf.	3	117± 7.5	14.85±1.14	2.94±.23	443±35.0	2.54± .25	.83±.13
Coron. insuf.	4	108± 6.0	12.60±1.45	3.65±.15	461±33.0	3.23± .23	.86±.04
Pulm. stenosis	4	133± 6.5	10.09±2.00	2.29±.01	231±44.0	1.8 ±0.10	.79±.41
Average	3	113± 7.4	11.72±1.10	3.28±.18	377±28.1	2.71± .33	.82±.15
		6.5%	9.4%	5.6%	7.5%	12.1%	17.8%
		Endurance	Oxygen Debt	Oxygen Transport			
Normal	4	10 ±0	8.5±7.6	3.56±.09			
Normal	8	10 ±0	7.3±3.4	4.16±.10			
Normal	5	10 ±0	11.1±4.0	3.68±.12			
Average		10 ±0	9.0±5.0	3.80±.10			
		0%	55.3%	2.7%			
Beryl. granul.	15	10 ±0	16.1±3.3	2.64±.19			
Beryl. granul.	18	9.7±1.2	10.9±3.9	2.93±.24			
Bronchostenosis	5	10 ±0	9.5±4.6	3.74±.13			
Pericardiectomy	4	10 ±0	7.8±2.6	3.68±.26			
Coron. insuf.	3	3.8± .3	38.0±6.4	3.78±.40			
Coron. insuf.	4	6.5± .9	21.0±1.7	4.23±.29			
Pulm. stenosis	4	5.3± .6	36.3±8.1	1.73±.25			
Average	3	7.9± .43	20.0±4.0	3.25±.25			
		5.4%	20.0%	7.7%			

Over-all average coefficient of variability: Normals 5%, Patients 10.2%

DISCUSSION

The variability, expressed in terms of per cent coefficients, for various respiratory and circulatory measurements has been determined by statistical analysis of the data obtained from the following: single exercise tests in 20 normal males and 15 normal females, and consecutive one minute observations of the primary measurements of heart rate, ventilation volume and respiratory efficiency, or volumes per cent of oxygen absorbed from expired air, including 17 repeated tests in normals, and 54 repeated tests in seven patients with definite disease entities. From these analyses there was no evidence of increased variability in the three primary measurements during exercise or recovery as compared with rest, and of these three, the respiratory efficiency showed the least variability. The overall average coefficient of variability for all factors of variability for each factor. Depending upon the level of significance one wishes to consider in evaluating differences in results between any two tests in the same patient, one may employ various multiples of the coefficient of variability. For example, since the range of plus or minus two standard deviations encompasses 95% of the possible values to be expected in a given representative sample, four times the coefficient of variability would approach an estimate of the range of non-significant variability for any measurement. In terms of exercise heart rate in patients with clinical disease, four times 6.5% would indicate 26% range of variation of exercise heart rate values which would be within the limits of variability. Hence a 30% change in heart rate, for the same individual, could be interpreted as being significant, i.e., beyond the range of variation of patient and testing procedure.

tors considered during exercise was about 12% in 35 normal subjects, and in repeated testing of either normals or patients with disease 5% or 10%, respectively. The factors which showed the least variation were, in addition to the duration of walking, the three primary measurements mentioned above, and the secondary measurements obtained by calculation, such as the oxygen consumption and oxygen transport per heart beat. When the three measurements of ventilation volume, oxygen consumption and oxygen removal or respiratory efficiency are considered for the 35 normal subjects, the average coefficient of variability is 13% during rest and 11.3% during exercise. These values are to be compared with those of Baldwin *et al.* who found 10% variation during rest in the basal state and 17% during one minute of exercise in 92 normal subjects (3).

In view of these findings, the technique of standardizing exercise tolerance testing described is considered satisfactory for the investigations of dyspnea in clinical patients. The essence of this technique is the combination of multiple continuous observations of responses to a standardized stress of exercise. The latter is controlled automatically by treadmill walking. The rate of energy expenditure is moderate, averaging just over twice the resting value, and the duration of 10 minutes is sufficiently long to screen out patients with circulatory insufficiency. Finally since the range of variation is less than previously reported values for observations in the basal state, it does not appear necessary to have this as a prerequisite to satisfactory testing technique when problems of metabolism are not under consideration. Fur-

thermore it permits observations of respiratory and circulatory performance to controlled work-loads under ordinary conditions of diet and preceding activity for the patients studied.

SUMMARY

1. A statistical analysis of the cardio-respiratory performance of 35 normal adults to a standardized stress of activity has been made to determine the normal limits and range of variability in the non-fasting state.

2. The average coefficient of variability for the several factors observed during rest, exercise, and recovery was about 12%.

3. Similar analyses of data from multiple tests on the same individual—either normal subjects or patients with clinical disease—showed average coefficients of variability of about 5% and 10%, respectively.

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STUDIES IN METHIONINE METABOLISM. II. FASTING PLASMA METHIONINE LEVELS IN NORMAL AND HEPATOPATHIC INDIVIDUALS IN RESPONSE TO DAILY METHIONINE INGESTION¹

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In earlier methionine studies (1), it was noted that the fasting plasma methionine⁴ levels in some patients with acute and chronic liver disease, receiving DL-methionine, tended to be markedly increased. It was not too clear in all instances whether this elevation related to the methionine administration, to the disease process, or to a combination of the two. It was definite, however, that severe liver disease in itself could account for some elevation of the fasting plasma methionine level. Over an 11-month period we have attempted further to clarify this picture.

PROCEDURES

The actual quantitative assay procedures have been previously described (2, 3). It might be emphasized here that one concept held earlier has been modified in that the normal fasting plasma methionine level varies within a very narrow range, from 0.25 mgm. per 100 cc. to 0.48 mgm. per 100 cc., with a mean of 0.33 mgm. per 100 cc. The previous concept of a wider normal range related to a longer period intervening between the obtaining of the blood sample and the preparation of the protein-free filtrate. Considerable proteolysis apparently occurs in a matter of a few hours in some plasma specimens. All determinations here reported have been performed on protein-free filtrates prepared within three hours from the time of withdrawal of blood.

The first patients and controls in this series were given 3 gms. of DL-methionine three times daily by mouth as compressed 0.5 gm. tablets, over varying periods of time. Prior to, during, and following such periods of adminis-

tration, fasting blood specimens were obtained for D- and L-methionine quantitation. In some of these individuals, urines collected over 24-hour and/or 72-hour periods were also assayed for D- and L-methionine.

When it became apparent that a striking difference existed in the methionine retention pattern between normal individuals and many patients with chronic liver damage, a standard program was set up consisting of the following procedures:

A. Studies prior to methionine administration:

1. Fasting blood specimens for plasma D- and L-methionine assay, Monday through Friday, during Week No. 1.

2. One or more three-day quantitative urine specimens during Week No. 1 for D- and L-methionine.

3. No medication of any sort during this period, except for routine "multi-vitamin" dietary supplementation in all patients,⁵ and parenteral vitamin K in those individuals with significantly low prothrombin levels. Such medication was constant throughout the entire period of study.

4. A diet high in protein, moderate in fat, and adequate in calories, was administered throughout the study. The most severe cirrhotics received a semi-liquid, high-protein, low-salt intake.

B. Studies during methionine administration:

1. On Sunday of Week No. 2, 3 gms. of DL-methionine were administered at 1:00 p.m. and 8:00 p.m.

2. Thereafter, 3 gms. of methionine were administered at 9:00 a.m., 1:00 p.m. and 8:00 p.m., daily during Weeks No. 2 and No. 3, up to and including 9:00 a.m. on Monday of Week No. 4.

3. Daily fasting bloods were obtained Monday through Friday for D- and L-methionine during Weeks No. 2 and No. 3.

4. Three-day quantitative urine specimens were obtained for methionine assay throughout this period.

C. Studies following the cessation of methionine administration:

1. After the 9:00 a.m. dose on Monday of Week No. 4, as previously noted, methionine was discontinued.

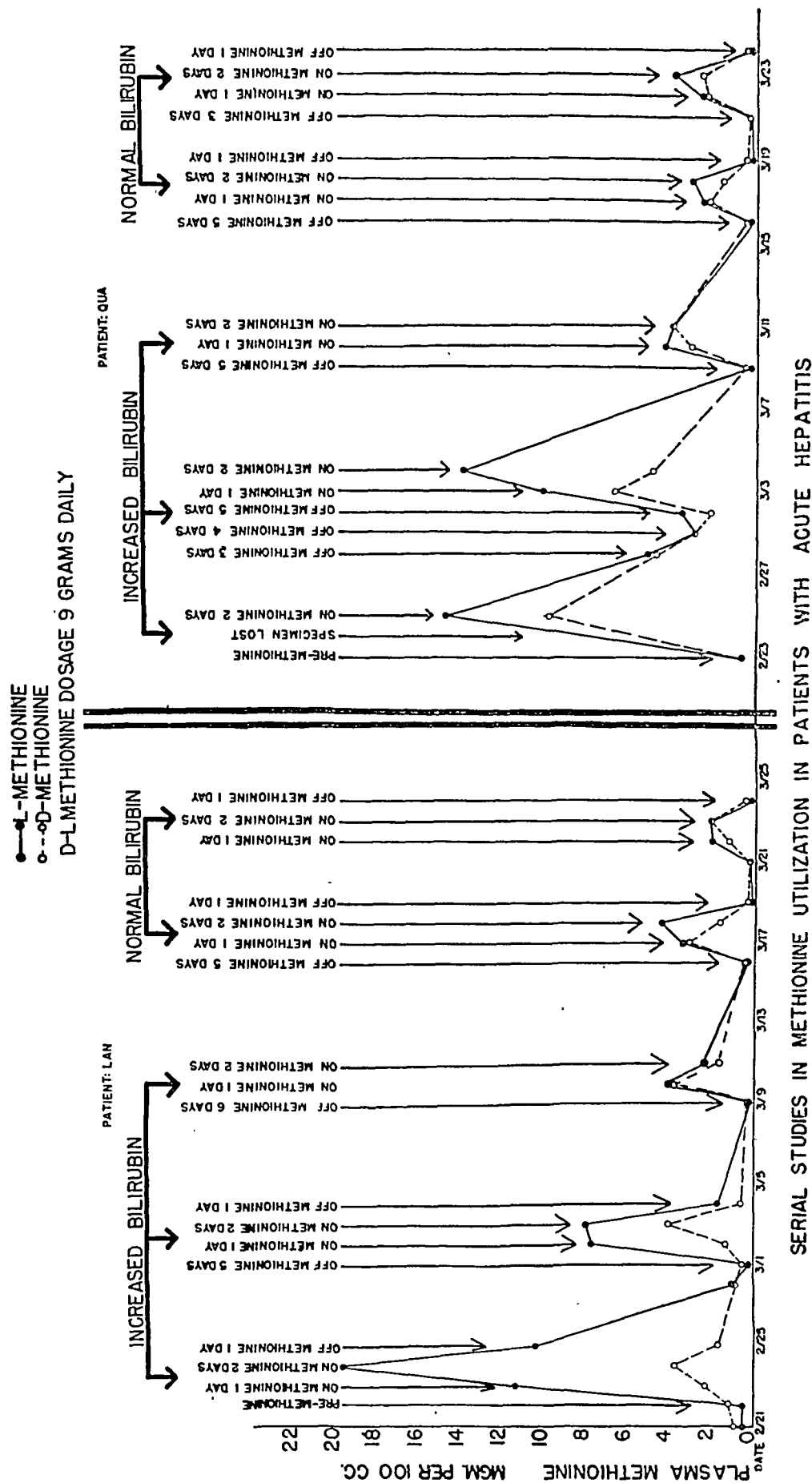
2. "Hexavitamin Tablets" (Strong, Cobb & Co., Inc.), six tablets daily.

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⁴ Unless otherwise qualified, the word "methionine" will refer to L-methionine, i.e., the natural isomer.



SERIAL STUDIES IN METHIONINE UTILIZATION IN PATIENTS WITH ACUTE HEPATITIS

FIG. 2. ACUTE HEPATITIS

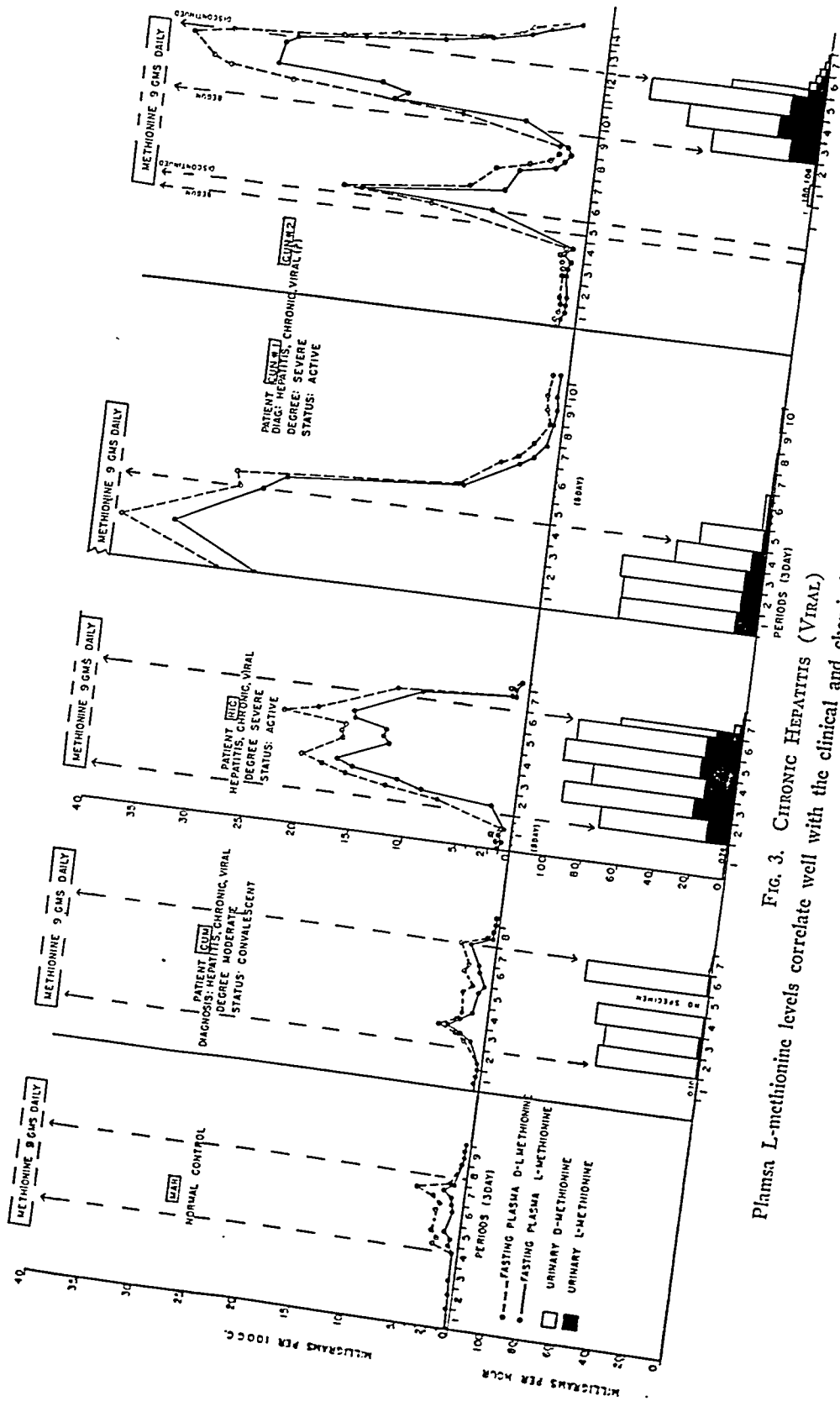


Fig. 3. CHRONIC HEPATITIS (VIRAL)
Plasma L-methionine levels correlate well with the clinical and chemical evidence of hepatic insufficiency.

tion (all of the usual tests were abnormal); protein metabolism (balance studies); and liver histology (biopsy). The histological findings are those of localized areas of round cell infiltration, evidence of hepatocellular abnormality and moderate amounts of periportal fibrosis. The actual proof of viral infection is unfortunately not possible.

Patient CUM, aged 25—At the time of this study his disease process appeared to be progressively less active, as evidenced by diminution in hepatomegaly (to approximately 3 cm. below the rib margin, on deep inspiration), gradual disappearance of spider angiomas, and diminishing abnormality of liver function tests.

Patient RIC, aged 21, had had involvement of the liver for a period of one and one-half or more years, dating from an attack of acute hepatitis prior to his original admission to this Hospital. At the time this test was performed, he had major abnormality of all liver function tests including a retention of 14 per cent bromsulfalein (5 mgm. per kgm. \times 45 min.). He also had evidence of portal hypertension including some splenomegaly, and had had bleeding from an esophageal varix about two months previously.

Patient CUN, aged 50, in the spring of 1946 noted gradual onset of painless jaundice. Six weeks later he was admitted to the Veterans Hospital in San Francisco at which time he had a major degree of ascites which required eight paracenteses. He was told at that time that he had viral hepatitis.

On March 1, 1948 he had a severe hemorrhage from esophageal varices. On admission to this Hospital ten days later, he was found to have considerable ascites, an enlarged, tender liver, moderate jaundice, and abnormality of all tests of liver function above noted. He was also found to have cardiac valvular disease probably referable to an old attack of rheumatic fever.

On a high-protein, adequate-calorie diet plus complete bed rest, he rapidly mobilized his ascitic fluid and showed considerable clinical improvement and some degree of improvement in liver function, although at no time did he have complete normality of any of the liver function tests used.

From March 18 to May 13, 1948, five fasting plasma methionine values were all significantly

above the normal, the highest being 1.51 mgm. per 100 cc.

On July 9, 1948 he was placed on 9 gms. of DL-methionine daily. By July 27 he had become obviously toxic, the toxicity including some degree of disorientation and a penetrating odor which was not entirely that of methionine and which suggested the so-called "hepatic fetor." The same dose of methionine, that is, 9 gms. daily, was continued until the 2nd of August, 1948. From July 21 until August 2, he also received 9 gms. of choline chloride daily. The first methionine value shown in Study No. 1 in this patient was obtained on July 21, that is, 12 days after the institution of methionine in a dose of 9 gms. daily, and on the first day of choline chloride administration.

The second methionine study was carried on as part of a protein balance study and was instituted on October 11, 1948. His liver function tests at this time were still significantly abnormal although he no longer had any detectable ascites. The initial portion of the second study was interrupted because of the appearance of symptoms which were attributed to salt deprivation referable to a period of hot weather and low-sodium intake. When this condition was corrected the study was resumed with the results shown in Figure 3. During this second period of methionine administration, he again manifested the symptoms of major and progressive mental clouding coupled with a strong fetor which was similar to, but probably not identical with that which is commonly called fetor hepaticus.

Following the completion of this balance study, the patient was permitted to return home and was instructed to come in for a further evaluation after the Christmas holidays. Unhappily, he had a fatal hemorrhage from esophageal varices about one month after leaving this hospital and his local physician obtained no postmortem examination.

The deviations from normal of the fasting plasma L-methionine values in all three patients are quite definite, and in the last two are most striking. The degree of abnormality would appear to parallel the severity of the disease process. It is also apparent that increased urinary L-methionine appears in those individuals with very high plasma levels.

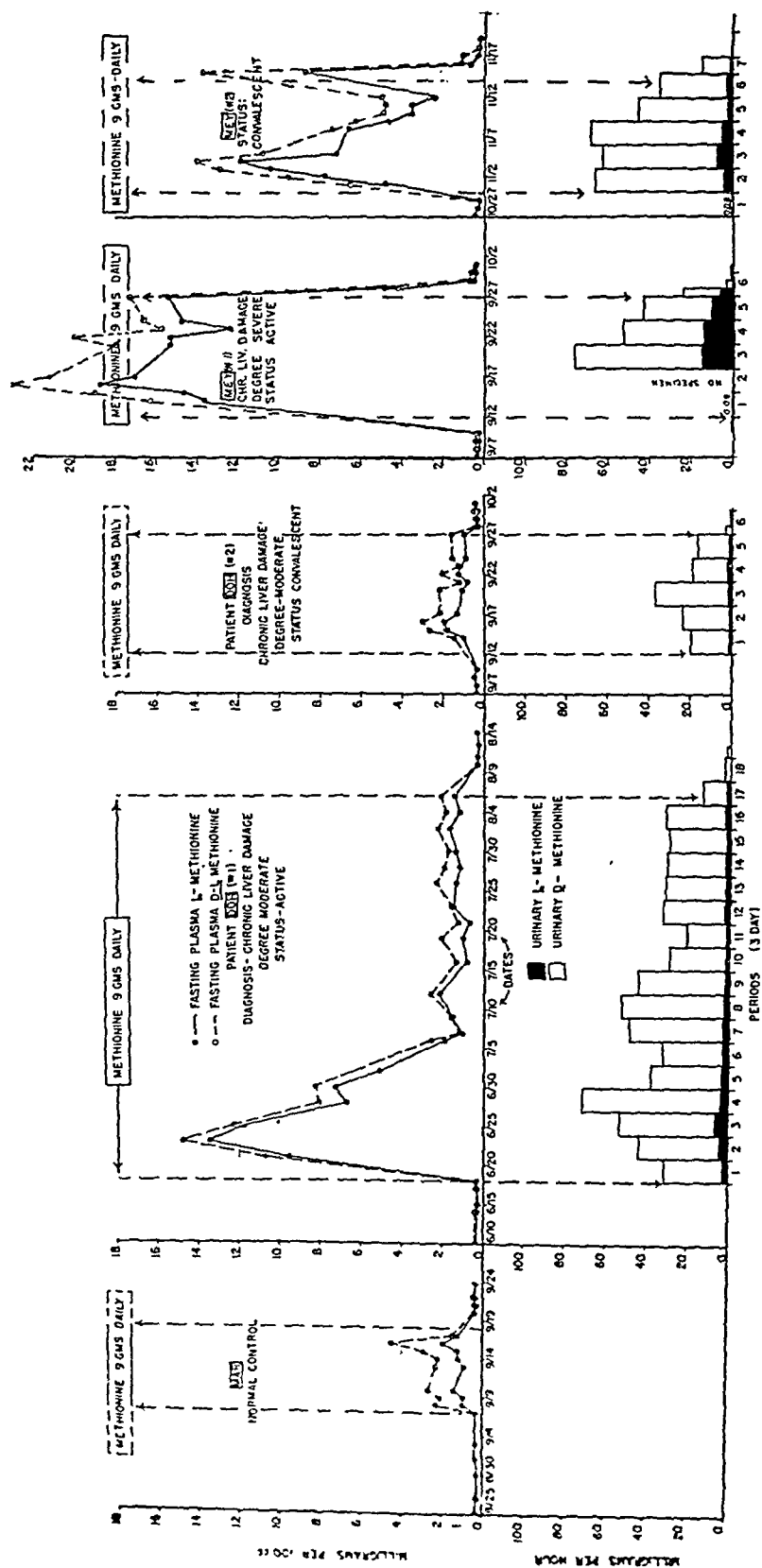


FIG. 4. CHRONIC NON-VIRAL HEPATITIS (CIRRHOSIS)

Methionine utilization improves as the liver becomes more competent.

methionine retention. He represents the only man so far with unquestionably active liver disease who has shown such a picture. It may be that hemachromatosis represents an extremely specific form of liver disease in which the utilization of methionine is not impaired. It should be noted,

however, that he was undergoing rapid clinical improvement referable to a high-protein, low-sodium intake, and hence that widespread protein tissue formation might account for such a finding.

Patient FER, aged 50, has chronic liver damage, presumably resulting from prolonged advanced nu-

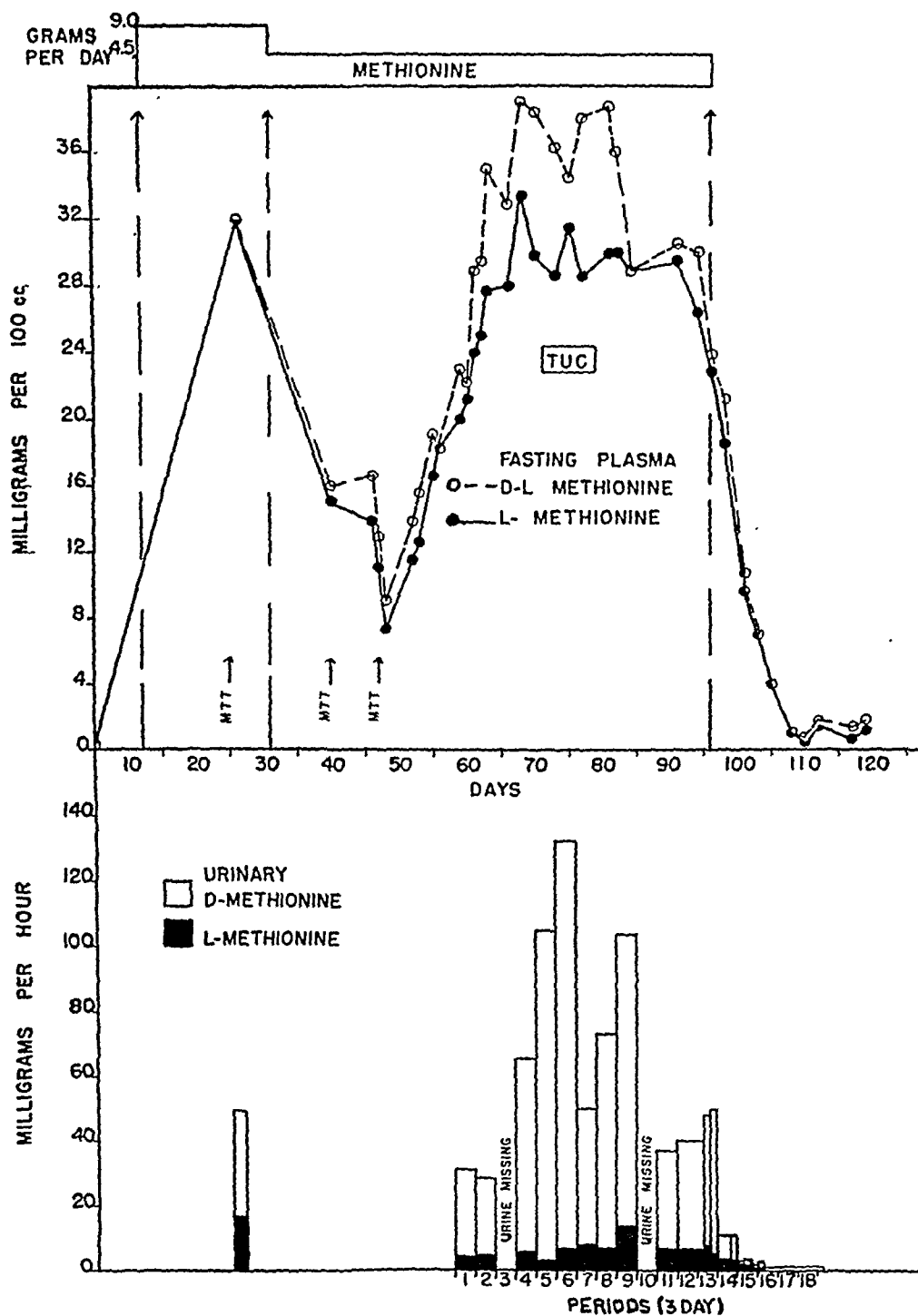


FIG. 6. PROLONGED METHIONINE STUDY IN PATIENT TUC (SEVERE HEPATIC INSUFFICIENCY)

Halving the dosage still resulted in marked elevation of the fasting plasma L-methionine. "MTT" refers to intravenous methionine studies (1).

tritional deficiency (he was a prisoner of war of the Japanese for four years). In addition to clinical and chemical evidence of moderate hepatic insufficiency, he has evidence of portal hypertension in terms of recurrent hemorrhages from esophageal varices and a considerable degree of splenomegaly. His plasma L-methionine pattern is similar to that seen in patients with moderate chronic liver damage.

One additional study (Figure 6) is included on a man with very severe cirrhosis, chiefly because of the length of the study and the fact that the dosage of methionine was diminished by one-half at one stage. It will be noted in this patient, TUC, that with reduction in dosage a temporary fall in the plasma methionine level occurred, but that with the continuance of the smaller dose a rise approaching the previous levels was obtained. The one high value on the dosage of 9 gms. of methionine was not entirely comparable with that observed in other individuals on the same dosage inasmuch as this fasting level was obtained as part of an intravenous methionine study and related to a discontinuance of orally administered methionine for a 48-hour period prior to the test. It is probable that had the fasting level been obtained without any such two-day interval, the value would have been higher than that noted in any other individual included in this report. The figure is presented chiefly to demonstrate that even moderate dosage of methionine in individuals with extreme degrees of hepatic insufficiency results in fasting blood levels which are quite unphysiologic.

DISCUSSION

From the preceding observations, it is apparent that many patients with liver damage are unable properly to utilize methionine, and that such impairment of methionine metabolism is no longer present when the liver damage disappears (in patients with acute hepatitis) or has become quiescent (in individuals with cirrhosis).

If one compares the abnormalities in plasma methionine in patients with liver disease with the blood sugar findings in diabetics, there appears to be some analogy. The individual with

severe liver disease has a high fasting plasma methionine level without previous methionine administration. The abnormality in methionine metabolism in the patient with mild or moderate liver damage is only apparent when stress is applied. In our experience thus far, a spontaneously high fasting level is always correlated with a prolonged, difficult convalescence, whether the disease be viral hepatitis or "cirrhosis."

The possible mechanisms of impairment in methionine utilization provide material for speculation. Three metabolic pathways are normally open: (1) Incorporation of the methionine into protein tissue; (2) demethylation with resultant homocysteine formation; and (3) oxidation of the methionine molecule with resultant excretion of the oxidized sulfur as urinary inorganic sulfate. It appears probable that the block occurs in (1) or (2), inasmuch as the excretion of urinary sulfate is considerably increased in some of the patients with high plasma methionine levels. Simultaneous quantitation of plasma cysteine will help to rule in or out the presence of block in the methionine \rightarrow homocysteine \rightarrow cysteine sequence. Such studies are under way at the present time.

Also in the process of evaluation is the determination of methionine utilization in patients with non-hepatic disease. Should a high degree of specificity for liver disease be found, it is conceivable that the procedure may have some clinical applicability.

The appearance of a reproducible picture of toxicity in patients whose ability to utilize methionine is impaired, suggests the need for caution in the indiscriminate use of this material as a therapeutic agent in individuals with liver disease. It is probably best to regard dietary methionine as the logical source of the amino acid in such patients.

SUMMARY

Daily administration of 9 gms. of DL-methionine to patients with liver damage results in a significant elevation of the fasting plasma L-methionine, as compared to normal controls; such abnormal retention disappears when the hepatic status reverts to normal.

In some patients with severe liver damage, continued administration of methionine results in toxic manifestations.

ACKNOWLEDGMENTS

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PROTAMINE (SALMINE) SULPHATE, HEPARIN, AND BLOOD COAGULATION

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The availability of standardized preparations of heparin and their widespread clinical use in the treatment of thromboembolism have prompted us to reinvestigate the action of protamine sulphate on the coagulation of blood and its use as an antagonist to heparin.

For the past three decades the coagulation mechanism of blood has been represented by two equations (1):

1. Prothrombin $\xrightarrow[\text{Calcium}]{\text{Thromboplastin}}$ Thrombin
2. Fibrinogen $\xrightarrow{\text{Thrombin}}$ Fibrin

An enormous amount of investigative work performed within the past few years has effected numerous modifications in these two equations. The present concept of the coagulation mechanism consists of a system of circulating proteins and other macromolecules exhibiting complex inter-relationships and antagonisms which become imbalanced during the process of coagulation. The results of experiments with individual protein inter-actions call upon the biochemist, physiologist, and clinician for concise interpretation and critical evaluation, and then upon repeated experimentation before conclusions can be accepted as fact for either theoretic or practical consideration.

The present investigation of protamine sulphate began as a clinical problem, but it was realized early that interpretation of the results depended upon concepts of the coagulation mechanism which are controversial. The purpose of this paper is to present a series of *in vivo* and *in vitro* experiments which extend the existing knowledge of the action of protamine sulphate on the coagulation mechanism.

EFFECTS OF SALMINE ON THE NORMAL CLOTTING MECHANISM

Effects of in vivo injections of protamine sulphate on clotting times of whole blood samples subsequently obtained

At the onset of this project, little was known concerning the possible toxic or side reactions of protamine when it was administered intravenously in normal non-heparinized patients.

It has been demonstrated (2) that large doses of protamine administered intravenously in the dog resulted in the formation of multiple thrombi (emboli). In these experiments on humans, therefore, 10 mg. of protamine were initially given and the dose progressively increased to 100 mg. For one hour following the intravenous injection, the blood pressure, pulse, and respiratory rate were recorded at 10-minute intervals. The coagulation time of the blood was determined by the Lee-White method before injection and at 10, 20, and 60 minutes afterward. Blood specimens were drawn from a three-way stopcock through which a slow intravenous infusion of normal saline was given during the course of the experiments. This was done to minimize the introduction of tissue thromboplastic substance into the test tubes. The stopcock was back washed with normal saline after the withdrawal of blood or the injection of heparin or protamine. The heparin in most instances was administered in the contralateral arm. Twenty seconds after clamping the infusion tube, a saline washed 5 ml. syringe was filled with blood and a stop watch started. Three saline rinsed 11 mm. test tubes were immediately filled with 1 ml. of blood. Coagulation was complete in each tube when the blood failed to flow on tilting with light tapping. The clotting times of the second and third tubes were averaged for recording. All determinations of clotting time were performed by the same technician. Results of these experiments are shown in Table I.

Topical application of protamine

Two dogs were anesthetized with intravenous sodium pentobarbital and areas 1 cm. square were abraded on the skin, liver, gastric serosa and mu-

TABLE I

Effect of intravenous protamine sulphate on the clotting time of whole blood

Subjects	Clotting time in minutes			
	Minutes after beginning experiment			
	0'	10'	20'	60'
10 Controls (Average)	16.6	15.2	14.2	11.0
Nine Experiments with 10-60 mg. Intravenous protamine (Average)	15.1	10.4	9.8	7.7
Acceleration of Clotting Time in 10 Controls (Average)		1.4	2.4	5.6
Acceleration of Clotting Time in nine Protamine Experiments (Average)		4.7	5.3	7.4

cosa. Bleeding from these areas was unaffected by the topical application of protamine when compared to adjacent control areas. Jorpes *et al.* (3) stated that protamine did not act as a local hemostatic agent in heparinized patients.

Effects of protamine on in vitro clotting systems

Whole blood

The effect of protamine on the clotting time of whole blood was observed by adding 2 ml. of whole blood immediately after withdrawal to increasing amounts of protamine sulphate. The clot-decelerating action of protamine is demonstrated in Table II. In all *in vitro* experiments the pH

TABLE II

Anticoagulant action of protamine on whole blood in vitro

Tube No.	Protamine sulphate*	Clotting time†	Ratio P/F‡
	mg.	minutes	
1		9	
2	.01	10.5	.0025/1
3	.02	9.5	.005/1
4	.1	10.5	.025/1
5	.2	11	.05/1
6	1.0	15	.25/1
7	2.0	19	.5/1
8	10.0	52	2.5/1
9	15.0	90 NC	3.7/1
10	20	90 NC	5/1

* To each tube containing protamine sulphate was added: 2 ml. whole blood + .85% NaCl to constant volume.

† NC = No clot.

‡ Ratio P/F = protamine/fibrinogen.

was 6.5 – 7.5 and the temperature 25° C. unless otherwise stated.

Fibrinogen-thrombin mixtures

Ferguson (4) previously pointed out a clot-accelerating effect of protamine when it was incubated with thrombin prior to the addition of fibrinogen. His work was corroborated. Experiments were then instituted to determine the effect of protamine on thrombin-fibrinogen mixtures in the presence of inactivated serum. Serum was inactivated by heating to 56° C. for 10 minutes in a water bath. Following inactivation, the serum was tested for fibrinogen, thrombin, and prothrombin by adding respectively thrombin, fibrinogen, and a mixture of calcium chloride, thromboplastin, and fibri-

TABLE III

Effect of protamine on thrombin in the presence of serum

Tube No.*	Protamine sulphate mg.	Clotting time† seconds
1		12.3
2	.02	13.7
3	.1	14.2
4	.2	13.8
5	.22	12.1
6	1.0	8.6
7	2.0	10.3
8†	4.0	41.2
9†	6.0	50 NC

* To each tube containing protamine sulphate was added: .1 ml. human inactivated serum + 5 u. thrombin + .85% NaCl to constant volume. After five minutes' incubation of the mixture, 1.65 mg. fibrinogen were blown in and the clotting time determined.

† NC = No clot.

‡ End point of clot formation indefinite.

nogen. No trace of fibrinogen, thrombin, or prothrombin was found. Serum inactivated in this manner does contain the heparin cofactor or substrate that heparin activates. This has been demonstrated elsewhere (5). Experiments were also set up to determine whether the five-minute incubation period produced any significant inactivation of the thrombin by the natural antithrombic activity of the serum. Control experiments, those with an incubation period of one minute and those with an incubation period of 11 minutes, produced clotting times of 12–13 seconds, on the addition of fibrinogen. Incubation periods beyond 10 minutes gave rise to more prolonged clotting times, up to 63 seconds after one hour of incubation.

There was no demonstrable accelerating effect of the protamine in tubes 2–5 (Table III). The

concentration of protamine in these tubes corresponded generally with that necessary to effect neutralization of .01 mg. of heparin *in vitro*. This is demonstrated subsequently in Table VII. In concentrations greater than this (tubes 6 and 7, Table III) there was a slight but significant reduction of the clotting time. Quantities of protamine greater than 2 mg. in this mixture (tubes 8 and 9, Table III) had a decided anticoagulant effect. In these two latter tubes, the ratio of protamine to fibrinogen was 2.4:1 and 3.6:1, and it will be shown that the inactivation of fibrinogen by the protamine produced this anticoagulant action.

Fibrinogen precipitation

Jaques (6) has shown that the protamine-heparin complex behaves according to the law of mass action, and he was able to demonstrate an equivalence point using whole dog blood. An excess of either heparin or protamine caused prolongation of the clotting time. The antithrombic (7-9) and antiprothrombic (10) action of heparin in the presence of a cofactor has been demonstrated, but the clot-decelerating action of protamine in whole blood is less well understood. According to Mylon *et al.* (11), protamine will precipitate plasma fibrinogen, and they suggested that its principal anticoagulant effect obtains by means of this precipitation. Protamine in concentrations of 1.5 mg., and 10 mg./ml. of plasma will precipitate 80 per cent and 100 per cent of the plasma fibrinogen respectively. In order to study this effect, the experiment displayed in Table IV was performed.

The addition of thrombin in reaction 2, Table IV, to the supernatant fluid without the development of a fibrin clot in 30 minutes indicates that the supernatant fluid from reaction 1 either has no fibrinogen in it, or if it is present it is bound to

TABLE IV

*Dissociation of a protamine-fibrinogen complex in the presence of serum**

1. 8.25 mg. Fibrinogen + 6 mg. Protamine Sulphate—White Ppt. + Supernatant.
2. .5 ml. Supernatant + 20 u. Thrombin—No Clot 30'.
3. .5 ml. Supernatant + 20 u. Thrombin—+ 1.65 mg. Fibrinogen—Clot 5''.
4. .1 ml. Serum + 20 u. Thrombin—No Clot 30'.
5. .5 ml. Supernatant + 20 u. Thrombin + .1 ml. Serum—Clot 5''.

* Fibrinogen 16.5 mg./ml. Protamine Sulphate 20 mg./ml. Thrombin 100 u./ml. Supernatant removed with a pipette.

TABLE V
Precipitation of fibrinogen by protamine in the presence of serum

Tube No.*	Protamine sulphate	Clotting time	Ratio P/F†
	mg.	seconds	
1		9	
2	2	5.3	.25/1
3	4	8.6	.5/1
4	6	17	.75/1
5	8	15-20†	1/1
6	10	No clot	1.25/1
7	12	No clot	1.5/1
8	14	No clot	1.8/1
9	16	No clot	2/1

* To each tube containing protamine sulphate was added: 8.25 mg. fibrinogen + .2 ml. human inactivated serum + .85% NaCl to constant volume + 20 u. thrombin and the clotting time determined.

† Fibrin strands only. End point indefinite.

‡ Ratio P/F = protamine/fibrinogen.

the protamine. When less than 6 mg. of protamine were used in reaction 1, a firm fibrin clot resulted from reaction 2. Reaction 3 precludes the destruction of thrombin by the supernatant fluid. Reactions 4 and 5, however, indicate that fibrinogen must have been present in the supernatant fluid and that in the presence of serum, dissociation of a protamine-fibrinogen complex presumably occurred and liberated sufficient fibrinogen to permit the formation of a fibrin clot. According to the mass action law, a higher concentration of protamine would further suppress the presumed dissociation of a protamine-fibrinogen complex and eventually result in complete fibrinogen precipitation even in the presence of serum. This is shown in Table V.

The approximate ratio of protamine to fibrinogen for complete inactivation in reaction 1, Table IV, was .7:1, while in the presence of serum the ratio is 1.25:1. Similarly, in plasma, the ratio is 2.5:1 (11). The ratio for whole blood has not been determined, but from the protamine to heparin titration tables, it appears that in tubes containing a protamine excess insufficient to inactivate all the fibrinogen the anticoagulant action could not be dependent on the partial inactivation of fibrinogen, since the clotting time is not necessarily a function of fibrinogen concentration. It will later be shown that the tubes containing a slight excess of protamine will not clot on addition of fibrinogen. Thus it was felt that the inactiva-

tion of fibrinogen was not the only acting anticoagulant action of protamine sulphate.

The above reactions were obtained with inactivated and active serum. The serum used did not clot within 20 minutes on addition of thromboplastin, calcium, and fibrinogen. Dissociation of the protamine-fibrinogen complex did not occur in solutions of thromboplastin or calcium chloride.

Character of the anticoagulant action of protamine, with special reference to antithromboplastic effects

Protamine may act as an antithromboplastin (12, 13) or an antiprothrombin (4, 12). To investigate these actions a series of *in vitro* experiments were carried out using whole blood. A quantity of protamine was used that would produce less than complete fibrinogen inactivation (Table VI).

Inactivation of the fibrinogen did not prevent clotting of the mixture of blood, sodium chloride, and protamine in tube 2 (Table VI) because the addition of an excess of fibrinogen in tube 3 had no significant effect. Further, the appearance of a normal fibrin clot in tubes 4 and 6 after the addition of thrombin showed that quantities of fibrinogen adequate for normal coagulation were present in solution. The results in tubes 7-12 indicated

TABLE VI

Character of anticoagulant action of protamine
Added after 30 minutes' incubation of basic mixture*

Tube No.	Thromboplastin†	Fibrinogen	Calcium chloride‡	Thrombin units	Clotting time	Type of clot
	mg.	mg.	mg.		minutes	
1	—	—	—	—	8§	Normal
2	—	—	—	—	> 120	
3	—	9.9	—	—	> 30	
4	—	—	—	20	20	Normal
5	—	—	.28	—	> 120	
6	—	—	.28	20 (after 80')	20	Normal
7	2.5	—	.28	—	60	Poor
8	5.0	—	—	—	9.6	Poor
9	7.5	1.65	—	—	5	Poor
10	12.5	—	—	—	2	Normal
11	2.5¶	—	—	—	42	Poor
12	5	—	—	—	12	Poor

* Basic mixture = 2 ml. whole blood + .2 ml. .85% NaCl + 16 mg. protamine sulphate.

† Thromboplastin (Maltine).

‡ Calcium chloride .025M.

§ Normal blood coagulation time. No protamine.

¶ Added immediately to basic mixture.

|| Incubated mixture of 5 mg. thromboplastin and 4 mg. protamine.

that protamine interfered with the conversion of prothrombin to thrombin except in the presence of an excess amount of thromboplastin. The conversion of prothrombin to thrombin is inhibited but not destroyed.

PROTAMINE-HEPARIN ANTAGONISMS

The treatment of thromboembolism with heparin is widely accepted at the present time. Although serious accidents from injudicious use of heparin are decidedly uncommon, they do occur and will become more frequent as heparin is more generally used and as preparations prolonging the anticoagulant effect of heparin are developed. Chargaff and Olsen (14), who reported the neutralization of heparin by protamine in 1937, recognized its potential clinical application, but until recently protamine has been used more in experimental than clinical medicine.

The toxicity of large intravenous doses of protamine has been demonstrated in rabbits (15), rats (2), mice (16), dogs (17), and guinea pigs (18). Jaques *et al.* (19), in 1938, and more recently Parkin and Kvale (18) and Cowley and Lam (20) showed absence of toxicity in dogs when smaller but effective doses were used. Jorpes (3), using up to 60 mg. of protamine intravenously, in 1939 demonstrated the antagonism of protamine (clupein) for heparin in humans. Allen (21) has given 2.5 mg./kg. in man without ill effects.

Different preparations of protamine vary in their capacity to neutralize heparin. The protamine used in these experiments was Lot No. 8738 obtained from the Upjohn Company.

Effect of intravenous protamine on heparinized patients

Time intervals, controls, and blood specimens were identical with those described previously. The three-way stopcock method of obtaining blood was utilized.

Fifty mg. of heparin¹ were administered intravenously after a preliminary clotting time had been done. Ten minutes later, the protamine in doses of 10, 30, and 50 mg. was given intravenously. In each instance, the anticoagulant ef-

¹ Abbott Laboratories.

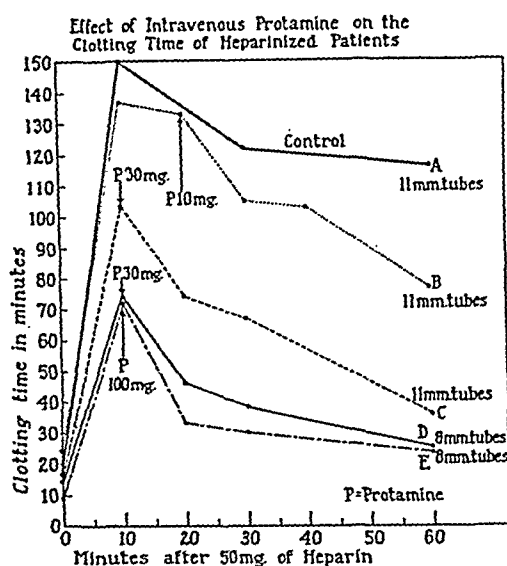


FIG. 1

effect of heparin was significantly reduced but not eliminated completely. Briefly, in 10 minutes 100 mg. of protamine produced approximately a 50 per cent decrease in the anticoagulant action of 50 mg. of heparin. The results in four cases are shown in Figure 1.

Protamine-heparin neutralization in whole blood

In order to verify the protamine-heparin ratio and investigate further the anticoagulant properties of protamine *in vitro*, titrations were carried out using normal blood. The same patient was used in all experiments.

Heparin, protamine, and normal saline were placed in

a clean dry test tube. Two ml. of freshly drawn blood were added to each tube and the clotting time determined by formation of a visible clot in the cork-stoppered tubes. At low heparin and protamine concentrations, the end point of coagulation was readily apparent, but in high concentrations some clot formation was evident before the entire solution was coagulated. The stop watch was started when blood first appeared in the syringe. Table VII shows the titrations.

The protamine-heparin ratio at neutralization is somewhat higher in the more concentrated mixtures, Titrations No. 2 and No. 3, but these compare favorably with the serum titrations. Although the higher concentrations of protamine would never be encountered *in vivo*, the zone of maximum neutralization is nicely demonstrated. This occurs because, except near the equivalence point, a relatively large excess of heparin or protamine is free to exert its anticoagulant action. In the latter two titrations, the difference between the control coagulation time and the coagulation time at neutralization is widened. In tube 9, Titration No. 1, the experiment was repeated using isotonic sodium sulphate instead of sodium chloride in a concentration corresponding with that in tube 10, Titration No. 3, and no significant change in the clotting time occurred. It may be presumed, therefore, that the concentration of the sulphate ion in tube 10, Titration No. 3, did not account for the elevated clotting time. No satisfactory explanation for this latter observation is apparent.

TABLE VII
 Protamine-heparin titrations using 2 ml. of heparinized whole blood *

Tube No.	Titration No. 1		Titration No. 2		Titration No. 3		P/H ratio
	Mg. protamine added to .01 mg. heparin	C. T.	Mg. protamine added to .5 mg. heparin	C. T.	Mg. protamine added to 1.0 mg. heparin	C. T.	
1	0	31	0	120 NC	0	120 NC	
2	.02	33	1	120 NC	—	—	2/1
3	.04	22	2	120 NC	4	120 NC	4/1
4	.06	22	3	120 NC	—	—	6/1
5	.08	18.8	4	120 NC	8	120 NC	8/1
6	.1	15.4	—	—	—	—	10/1
7	.12	14.1	6	120 NC	12	120 NC	12/1
8	.14	11.7	—	—	14	120 NC	14/1
9	.16	14.3	8	14.5	16	63	16/1
10	.18	18.8	—	—	18	39	18/1
11	.2	19	10	15	20	43	20/1
12	—	—	12	21.5	24	88	24/1
13	—	—	14	38	28	89	28/1
14	—	—	16	120 NC	—	—	32/1
15	Control	10.5	Control	10	Control	11	

*.85% NaCl added to constant volume. C. T. = Clotting time in minutes. NC = No clot.

Effects of protamine on heparin plus the serum heparin cofactor, tested on thrombin-fibrinogen mixtures in vitro

Experiments were carried out to determine the protamine-heparin ratio that would effect neutralization of the latter in the presence of the serum heparin cofactor. A thrombin-fibrinogen mixture was used. Inactivated serum, previously referred to, was used in order to eliminate any antithrombin, antithromboplastic, or fibrinogen inactivating effect of the protamine. Serum inactivated by heating at 56° C. for 10 minutes does not lose significant amounts of its heparin cofactor activity (5). Titrations are shown in Table VIII and Figure 2.

The addition of the protamine to the heparin first or to the serum first did not alter the final clotting times. Under normal circumstances over 90 per cent of the thrombin incubated with the serum and heparin will be destroyed in five minutes (5). The coagulation time of the system depends, in the absence of protamine, on the thrombin remaining after its partial destruction by the antithrombic activity of serum cofactor activated by heparin. An 18:1 ratio of protamine to

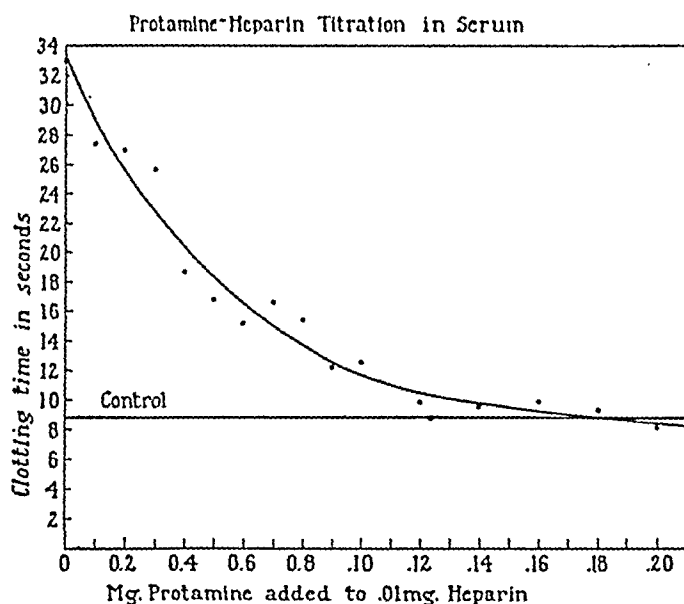


FIG. 2

heparin effected neutralization in repeated titrations. Increasing the ratio to 200:1 resulted in a slightly greater decrease in the coagulation time.

Action of thromboplastin on in vitro clotting of whole blood in the presence of an excess of heparin or protamine

Experiments were set up to extend the observations made in Table VI on the effect of protamine and heparin on whole blood *in vitro* in the presence of an excess amount of thromboplastin. It had been noted in other experiments using recalcified plasma that the incubation of protamine with thromboplastin did not reduce the clot accelerating effect of the thromboplastin alone. Tubes 1, 4, 7, and 14, Titration 2, Table VII, were repeated. After observing no clot for two hours an excess of thromboplastin was added to each tube. No clot occurred in tubes 1, 4, and 7, but tube 14 clotted in 70 seconds. In tube 14 the heparin had apparently been neutralized and coagulation was inhibited by the excess of protamine which was overcome by the thromboplastin added.

DISCUSSION

There has been no observation in the literature to the effect that the intravenous administration of protamine will produce a shortening of the clotting time of the blood. Chargaff (14) reported that he was able to confirm the observation of Waldschmidt-Leitz and collaborators (22) that protamine had an inhibiting effect on blood clotting

TABLE VIII

Protamine-heparin titration in serum

Protamine sulphate* mg.	Clotting time seconds	Ratio P/H§
—	8.8†	
—	32.7†	
.01	26.8	1/1
.02	27.4	2/1
.03	25.8	3/1
.04	18.5	4/1
.05	16.7	5/1
.06	15.3	6/1
.07	16.3	7/1
.08	15.3	8/1
.09	12.1	9/1
.1	12.5	10/1
.12	9.9	12/1
.14	9.6	14/1
.16	9.8	16/1
.18	9.3	18/1
.2	8.2	20/1
.4	9.5	40/1
.8	7.6	80/1
1.2	7.3	120/1

* To each tube containing protamine sulphate was added: 1 ml. human inactivated serum + .01 mg. heparin + 8 u. thrombin + .85% NaCl to constant volume. After five minutes' incubation of the mixture, 1.65 mg. fibrinogen was blown in and the clotting time determined.

† Control clotting time without heparin or protamine.

‡ Clotting time on addition of .01 mg. heparin.

§ Ratio P/H = protamine/heparin.

in vitro, but in dogs intravenous doses up to 11 mg./kg. failed to show any deviation of the clotting time from controls. Chargaff (23) later found that protamine did not alter the coagulation time of chicken plasma. Jaques (6) added protamine (.017 to 1.0 mg.) to 1 ml. of dog blood and demonstrated an anticoagulant effect with the higher concentrations. Cowley and Lam (20) reported a decreased coagulation time in a dog 30 minutes following 200 mg. of protamine, but no such effect was encountered with smaller quantities of protamine. The results of the experiments reported in this paper show a slight clot-accelerating "tendency" after the intravenous administration of protamine. Although the clotting times were performed with the utmost care in all experiments, it is our belief that technical variations account for the difference in clotting times between the controls and the protamine experiments. The only conclusion that can be arrived at, in accordance with the views of other investigators in this field, is that the intravenous administration of protamine has no clot-accelerating action in the absence of heparin.

The clot-decelerating effect of protamine on whole blood *in vitro* was demonstrated in Table II. In these experiments, there was no significant effect on the addition of protamine as long as the concentration of protamine corresponded to that found in whole blood after the intravenous administration of 100 mg. or less. As the protamine concentration *in vitro* is increased, however, there is a progressive prolongation of the clotting time. Although protamine in sufficient concentration will prevent clot formation by inactivating fibrinogen, the progressive prolongation of the clotting time in these experiments was not due to such inactivation since in few of the tests did the concentration of protamine in 2 ml. of whole blood attain high enough values. Since the rapidity with which coagulation occurs depends to a great extent on the amount of thrombin present, it appears that the anticoagulant effect of the protamine in these experiments was due to impaired thrombin formation.

Mylon, Winternitz, and de Sütö-Nagy (11) demonstrated that protamine will precipitate fibrinogen. The experiments performed and indicated in Tables IV and V demonstrate that protamine precipitates fibrinogen but in addition forms a

soluble fibrinogen-protamine complex that in the presence of inactivated serum makes available sufficient fibrinogen to allow coagulation on the addition of thrombin. When the concentration of protamine is increased, dissociation of the fibrinogen-protamine complex is inhibited and coagulation prevented. When referring to the effect of protamine on the second stage of coagulation, we prefer to use the term "protamine inactivating effect on fibrinogen" rather than "protamine precipitating effect."

Experiments listed in Table VI suggest that protamine interferes with the conversion of prothrombin to thrombin in whole blood except in the presence of an excess amount of thromboplastin. Tubes 2, 7, 8, and 12 indicate that the interference with the conversion of prothrombin to thrombin by the protamine is not an antithromboplastic effect. Conclusions beyond these cannot be drawn without determining the effect of protamine on a clotting system containing purified prothrombin.

The *in vivo* effect of protamine on heparinized patients duplicated the results of previously reported observations by others and needs no comment. It should be stated, however, that any clinician using protamine to neutralize heparin *in vivo* should be thoroughly acquainted with the character and strength of the protamine preparation he is utilizing. Preparations may vary considerably in their capacity to neutralize heparin. It also appears that the neutralization of a certain quantity of heparin *in vivo* may require considerably less protamine than is necessary for neutralization *in vitro*.

The neutralization of heparin by protamine in whole blood *in vitro* is listed in Table VII. Neutralization was effected with a protamine-heparin ratio of approximately 16:1. The protamine used in these experiments was Lot No. 8738 (Upjohn). A preparation subsequently obtained (Lot No. 8960 Upjohn) showed an *in vitro* protamine-heparin ratio of 0.7:1.

The neutralization of heparin was also determined in an isolated clotting system containing fibrinogen, thrombin, the serum heparin cofactor, heparin, protamine, and saline (Table VIII). Neutralization was complete at a protamine-heparin ratio of 20:1, which agrees satisfactorily with the 18:1 ratio observed in whole blood (Table VII). It is apparent from the observa-

tions made in these experiments plus those made from the same isolated system without the addition of heparin that protamine has no significant effect upon the heparin cofactor.

SUMMARY

1. Protamine sulphate administered intravenously in doses up to 100 mg. in humans has no effect upon the clotting time of whole blood.

2. Protamine sulphate had no effect as a local hemostatic agent in dogs.

3. In relatively low concentrations in inactivated serum, protamine has a clot-accelerating effect on a thrombin-fibrinogen mixture after incubation of the thrombin with protamine.

4. The antagonism to heparin was demonstrated *in vitro* and *in vivo*. In the presence of serum or whole blood, the protamine-heparin ratio at neutralization was 18:1. An excess of either caused prolongation of the coagulation time.

5. When present in whole blood in concentration insufficient to cause complete inactivation of fibrinogen, the anticoagulant effect of protamine is due to its interference with the conversion of prothrombin to thrombin. This antiprothrombic effect is not apparent in the presence of an excess of thromboplastin.

6. An antithromboplastic action of protamine sulphate could not be demonstrated.

7. Protamine does neutralize the serum heparin cofactor.

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THE EFFECT OF THEOPHYLLINE ETHYLENE DIAMINE ON RENAL FUNCTION IN CONTROL SUBJECTS AND IN PATIENTS WITH CONGESTIVE HEART FAILURE¹

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The importance of a renal factor in the pathogenesis of peripheral cardiac edema has been emphasized in recent publications (1-11). The work of Fitcher and Schroeder (1) indicated that the renal excretion of salt and water was impaired during cardiac decompensation. Numerous other workers (4-6, 10, 11) reported a reduction in renal blood flow, glomerular filtration rate, and sodium excretion in cases of cardiac failure. These studies resulted in the formulation of two principal hypotheses to explain the retention of sodium and water; namely, (1) low glomerular filtration rate, and (2) more complete reabsorption of sodium and water. More recently, Wesson, Anslow, and Smith (12) have suggested that the low filtration rate delivers a sodium load to the distal tubule which is less than the T_m for sodium and leads to more complete distal tubular reabsorption of sodium.

The purpose of this investigation was to study the renal mechanism of sodium and water excretion in cardiac failure by observing the effect of theophylline ethylene diamine (T.E.D.) on filtration rate, effective renal plasma flow, and sodium clearance. Since the results of preliminary experiments suggested that T.E.D. not only increased the tubular load of sodium but also decreased sodium reabsorption, the relationship between tubular load and excretion of sodium could not be determined. However, the simultaneous measurement of filtration rate and sodium clearance during the control periods in patients with

cardiac failure afforded an opportunity to study the degree of correlation of these functions under resting conditions. Since the evidence for the mechanism of xanthine diuresis in man is conflicting (4, 11), an extensive study of control subjects was made.

MATERIALS AND METHODS

Subjects

Male subjects were selected from the wards of the Baltimore City Hospitals and Infirmary (Home for the Aged).

Control subjects were chosen on the basis of a history, physical examination, chest roentgenogram, and urinalysis; cases with hypertension, cardiac and renal diseases were excluded. Although they were selected irrespective of age, most of them were over 50 (range = 36-78, mean = 60). All subjects were on a regular diet without salt restriction.

The patients in congestive heart failure were selected on the basis of clinical findings characteristic of cardiac decompensation. The majority had experienced repeated episodes of cardiac failure. Individual protocols are as follows:

Case 1, C. M. A 63-year-old colored male was readmitted with congestive heart failure for the third time on 10/30/47. When renal function was studied on 2/26/48, physical examination revealed a pulse of 88, a blood pressure of 132/84, and a respiratory rate of 30. He was dyspneic, orthopneic, and edematous. Moist râles were present at both lung bases. A harsh systolic murmur was audible at the apex. An X-ray of the chest revealed generalized cardiac enlargement and pulmonary congestion. An electrocardiogram showed myocardial disease and a tendency toward right axis deviation. The diet was regular. Treatment consisted of digitalis and mercupurin; the last injection of mercupurin was nine days prior to the experiment.

Case 2, J. P. A 43-year-old white male entered the hospital with cardiac decompensation for the third time on 3/5/48. Five weeks prior to the present admission he discontinued digitalis; signs of left and right heart failure ensued. On the day of the renal function studies, 3/8/48, physical examination revealed a pulse of 80, a blood pressure of 120/88, and a respiratory rate of 22.

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He was dyspneic and orthopneic, and râles were present at both lung bases. A harsh, apical, systolic murmur and a localized, apical, mid-diastolic murmur were present. An X-ray of the chest revealed cardiac enlargement with a typical mitral configuration and pulmonary congestion. An electrocardiogram showed auricular fibrillation, myocardial disease, and right axis deviation. He received a Karrell diet, and 2 cc. of mercupurin were given two days before the experiment.

Case 3, A. W. A 39-year-old colored male entered the hospital with cardiac failure for the seventh time on 3/17/48. Physical examination on the day of the experiment 3/22/48 revealed a pulse of 100, a blood pressure of 120/80, and a respiratory rate of 32. He was dyspneic and orthopneic. Moist râles were present throughout the lower portion of both lungs. A loud, harsh, moderately high-pitched, systolic murmur and a short, low-pitched, pre-systolic murmur were audible at the apex. An X-ray of the chest revealed cardiomegaly with a mitral configuration and pulmonary congestion. An electrocardiogram showed left bundle branch block, myocardial disease, and ventricular premature contractions. The liver was enlarged; ascites was present; but pitting edema of the lower extremities was minimal. He received a regular diet, and on 3/18/48, 2 cc. of mercupurin were given.

Case 4, R. P. An 82-year-old white male entered the hospital from the infirmary for the first time on 4/28/48 because of dyspnea of several weeks' duration. When the experiment was performed the following day, physical examination revealed a pulse of 96, a blood pressure of 210/100, and a respiratory rate of 28. Râles were present at both lung bases. An X-ray of the chest revealed left ventricular enlargement with pulmonary congestion. The second aortic sound was accentuated; no murmurs were heard. An electrocardiogram showed

myocardial disease and left axis deviation. There was pitting edema of the lower extremities. The diet was regular. He had not received treatment for cardiac failure.

Case 5, F. B. A 76-year-old white male entered the hospital from the infirmary on 2/28/48 with dyspnea of two months' duration. On the day of the experimental study, 3/1/48, physical examination revealed a pulse of 84, a blood pressure of 160/95, and a respiratory rate of 20. The eyegrounds showed evidence of arteriosclerosis. The radial and brachial arteries were thick and tortuous. Râles were present at both lung bases. The chest was emphysematous, and the heart sounds were faint. No murmurs were heard. A chest film revealed cardiac enlargement, the aorta was elongated and tortuous, and calcification was noted in the aortic knob. An electrocardiogram showed auricular fibrillation, left bundle branch block, myocardial disease, and left axis deviation. The lower extremities were markedly edematous. He had been on a regular diet and had not received therapy for cardiac failure.

Case 6, B. S. The patient was a 76-year-old white male who had been in the chronic hospital for several months with mild cardiac decompensation. When renal function studies were performed on 3/4/48, physical examination revealed a pulse of 80, a blood pressure of 160/60, and a respiratory rate of 20. The eyegrounds showed evidence of arteriosclerosis. Localized plaques were palpable in the radial arteries. Râles were present at both lung bases. The heart was enlarged to the left anterior axillary line. A loud systolic murmur was heard over the entire precordium, and an aortic diastolic murmur was transmitted to the apex. An electrocardiogram showed right atypical bundle branch block, myocardial disease, and ventricular premature contractions. There was pitting pedal edema. The serologic test for

TABLE I
(Experiments I and II)
The effect of theophylline ethylene diamine on renal function in control subjects

		Control periods				Periods during or after administration of theophylline ethylene diamine				
		1	2	3	Average	4	5	6	7	8
Glomerular Filtration Rate <i>cc./min./1.73 sq.m.</i>	A*	87.3	91.9	84.7	87.2	101.0	104.9	102.8	99.1	100.8
	B†	81.7	87.2	82.3	83.7	107.9	99.6	99.3	103.0	98.7
Effective Renal Plasma Flow <i>cc./min./1.73 sq.m.</i>	A	403.7	422.1	393.6	405.0	436.8	428.0	417.9	397.4	400.1
	B	371.3	387.0	376.4	378.2	503.9	421.6	398.4	435.1	407.7
Filtration Fraction <i>per cent</i>	A	22.0	22.2	21.6	21.8	23.4	24.8	24.9	25.3	25.4
	B	22.2	23.0	22.1	22.5	21.9	24.1	25.7	23.9	24.5
Urine Flow <i>cc./min./sq.m.</i>	A	1.72	2.60	2.51	2.41	7.38	5.74	5.44	4.78	4.24
	B	2.39	2.73	2.66	2.59	9.15	7.31	5.38	5.36	4.39
Sodium Excretion <i>m.eq./min.</i>	B	.266	.291	.280	.279	1.016	1.070	.880	.928	.819

* A. Single dose of 0.48 gm. T.E.D.; urine collections interrupted during injection. (Mean values for nine subjects.)

† B. 0.48 gm. T.E.D. injected during first five minutes of period 4; 0.48 gm. T.E.D. infused during remainder of experiment. (Mean values for six subjects.)

TABLE II
(Experiment III)*The effect of a single injection of theophylline ethylene diamine on renal function in control subjects (N = 10)*

		Control periods				Periods after administration of theophylline ethylene diamine				
		1	2	3	Average	4*	5	6	7	8
Glomerular Filtration Rate cc./min./1.73 sq.m.	Mn	97.1	98.4	94.3	96.6	119.4	102.9	105.9	107.4	106.1
	σ_{Mn}	6.6	6.1	6.8	6.1	7.2	6.5	7.4	6.4	6.4
Effective Renal Plasma Flow cc./min./1.73 sq.m.	Mn	469.6	481.7	472.4	474.6	618.9	475.5	470.7	455.9	462.2
	σ_{Mn}	46.6	37.2	43.0	40.6	55.8	37.1	40.4	38.5	40.4
Filtration Fraction per cent	Mn	21.4	20.9	20.4	20.9	19.9	22.0	23.0	24.2	23.6
	σ_{Mn}	1.1	0.8	0.9	0.9	1.1	0.9	0.8	1.1	1.0
Urine Flow cc./min./sq.m.	Mn	2.32	2.62	2.59	2.51	8.20	5.19	3.88	3.44	3.16
	σ_{Mn}	.5	.4	.4	.4	1.0	.5	.6	.3	.4
Sodium Clearance cc./min.	Mn	2.26	2.33	2.25	2.28	7.32	6.88	6.35	6.61	6.03
	σ_{Mn}	.25	.21	.26	.23	.78	.48	.49	.59	.40
Concentration of Sodium in Urine m.eq./cc.	Mn	.089	.087	.081	.086	.075	.114	.153	.159	.170
	σ_{Mn}	.018	.014	.014	.014	.006	.012	.022	.014	.019

* Injection of 0.48 gm. of drug during the first five minutes of the fourth clearance period

syphilis was positive. Treatment had consisted of 2 cc. of mercupurin weekly; no digitalis was taken. His last injection of mercupurin had been on 2/27/48. His diet was regular.

Experimental procedure

The test was begun after a 12-hour fast at 8:00 a.m., and three control clearance periods were usually completed by 9:30 a.m. Each subject was given 100 cc. of water at 6:00 a.m., and every one-half hour thereafter until the control periods had been completed. Filtration rate and effective renal plasma flow were measured by inulin and diodrast clearance respectively (13). All tests were made at constant blood levels of inulin and diodrast, using an infusion rate of 3.0 cc. per minute. Urine samples were collected by catheterization over 10-12 minute periods.

Venous pressure was measured by the method of Moritz and von Tabora and was referred to a point 10 cm. from the back at the level of the fourth intercostal space adjacent to the sternum.

Experimental Design—Control Subjects

A preliminary study (Experiments I and II) was conducted to determine a satisfactory time schedule and dose of intravenous T.E.D. Experiment III served as the control series for the study of patients in cardiac failure.

Experiment I. The effect of a single injection of T.E.D. on renal function with interruption of clearance periods during the injection. At the completion of three control clearance periods of 10-12 minutes each, 0.48 gm. of T.E.D. was administered intravenously over a period of five minutes. At the end of the injection, the bladder was washed, and five more clearance periods were fol-

lowed. Only inulin and diodrast clearances were determined in the nine subjects of this experiment.

Experiment II. The effect of the continuous administration of T.E.D. on renal function. In order to increase the magnitude of the effect, a larger dose (0.96 gm.) of the drug was given in this experiment. A single injection of 0.48 gm. of T.E.D. was administered intravenously during the first five minutes of period 4 and another 0.48 gm. was infused with the inulin and diodrast solution during the remaining five clearance periods. In this group of six subjects, sodium excretion was studied in addition to the clearance of inulin and diodrast.

Experiment III. The effect of a single injection of T.E.D. on renal function without interruption of the clearance periods during the injection. On the basis of the results of Experiments I and II, a single injection of 0.48 gm. of T.E.D. was made during the first five minutes of clearance period 4 in ten subjects. Inulin, diodrast, and sodium clearances were determined.

Experimental Design—Patients in Congestive Heart Failure

Experiment IV. The same time schedule and dose of T.E.D. as in Experiment III (control group) were used in the study of the six patients in heart failure.

Chemical Methods

Inulin analyses were made according to the method of Harrison (14). A modification of the method of Flox, Pitesky, and Alving (15) was used in the determination of diodrast iodine (16). It was demonstrated that T.E.D. did not interfere with the chemical recovery of inulin and diodrast iodine from human plasma. So-

dium was determined gravimetrically according to the method of Butler and Tuthill (17).

RESULTS

Control subjects

(Experiments I, II, III)

Glomerular filtration rate. Administration of T.E.D. was followed by a sustained elevation of the glomerular filtration rate which persisted for 55–60 minutes in the control subjects (Tables I and II) (average control period—period 8; $t = 3.64$, $P < 1\%$, $N = 10^3$ for Experiment III). The magnitude of the rise varied with the dose of T.E.D. so that the greatest increase occurred with the continuous infusion of 0.96 gm. (Experiment II).

Effective renal plasma flow. The response of effective renal plasma flow to T.E.D. was variable. In some cases, a transitory rise was observed during the fourth clearance period only; during the remaining periods effective renal plasma flow was at a control level or below. In other subjects, renal plasma flow increased during the fourth clearance period and gradually returned to the control value during periods 5–8. The average change for 10 subjects (Experiment III) was an elevation during the fourth period only (Table II, Figure 1) (average control period—period 4; $t = 5.64$, $P < 1\%$, $N = 10$) (average control period—period 8; $t = .78$, $P > 90\%$, $N = 10$). After continuous infusion of 0.96 gm. of T.E.D., the elevation in effective renal plasma flow was consistently maintained for a longer period than after a single injection.

Filtration fraction. The average filtration fraction remained unchanged during the fourth and fifth clearance periods but increased significantly

in the remaining three periods (6–8) (average control period—period 5; $t = 1.34$, $P = 90\%$, $N = 10$) (average control period—period 8; $t = 5.15$, $P < 1\%$, $N = 10$). Wide individual differences in response were observed.

Urine flow. Average urine flow increased threefold immediately after the administration of T.E.D. (average control period—period 4; $t = 6.62$, $P < 1\%$, $N = 10$) and gradually returned to a level slightly above the control values in the remaining periods (average control period—period 5; $t = 4.77$, $P < 1\%$, $N = 10$) (average control—period 8; $t = 1.44$, $P = 80 - 90\%$, $N = 10$).

Sodium clearance. The average sodium clearance (Experiment III) increased approximately threefold for 55–60 minutes after the administration of T.E.D. (average control period—period 4; $t = 6.72$, $P < 1\%$, $N = 10$) (average control period—period 8; $t = 8.72$, $P < 1\%$, $N = 10$). The mean plasma sodium values for the 10 cases were 139.7, 139.1, 139.5, 138.3, 138.8, 137.9, 139.2, and 137.9 m.eq./L. for clearance periods 1–8 respectively. The difference between the average control value for plasma sodium and the average for the five periods after the injection of T.E.D. was insignificant ($t = 2.58$, $P = 4\%$, $N = 10$). The concentration of sodium in the urine remained unchanged during the fourth clearance period but increased gradually during the remaining periods until it doubled the control value (average control period—period 4; $t = 0.70$, $P > 90\%$, $N = 10$) (average control period—period 8; $t = 3.65$, $P < 1\%$, $N = 10$).

Venous pressure. Venous pressure decreased from a mean control value of 10.0 to 8.3 cm. of saline after the administration of T.E.D. ($t = 9.08$, $P < 1\%$, $N = 10$).

Patients in congestive heart failure

(Experiment IV)

The six cases in this experiment included two patients with inactive rheumatic valvular disease, two with arteriosclerotic heart disease, one with hypertensive cardiovascular disease, and one with syphilitic and arteriosclerotic heart disease. The findings from the study of two of these cases which illustrate the variation in the control values and the response in renal function to T.E.D. are compared with the average values for the control

³ Since all values obtained before and after the administration of T.E.D. in a given subject are highly correlated, all tests for significances were made by calculating t scores by the difference method.

$$t = \frac{\frac{\sum(x_1 - x_2)}{N}}{\sigma_{\text{Mn diff.}}} = \frac{\text{Mn diff.}}{\sigma_{\text{Mn diff.}}}$$

$$\sigma_{\text{Mn diff.}} = \frac{\sigma_{\text{diff.}}}{\sqrt{N - 1}}$$

$$\sigma_{\text{diff.}} = \sqrt{\frac{\sum(x_1 - x_2)^2}{N} - \left(\frac{\sum(x_1 - x_2)}{N}\right)^2}$$

where x_1 and x_2 are observations made before and after treatment in the same subject.

group (Experiment III) in Figure 1. The results for the entire group are summarized in Table III.

Control periods. The mean values for the three control clearance periods for filtration rate varied from normal to more than 50% below normal. Effective renal plasma flow was reduced below 300 cc./min./1.73 sq.m. in all cases. Filtration fraction was elevated in all but Case 6. Sodium clearance was reduced considerably below the mean value for the control group (2.28 cc./min. \pm .23) in Cases 1-4 but was elevated in Cases 5 and 6. Attention is called to the fact that a low sodium

clearance was present in cases with a normal filtration rate while the cases with an elevated sodium clearance showed a low filtration rate. Urine flow was diminished below the average control level in all but Case 5. The concentration of sodium in urine was lower than the average control level in all but Case 6.

Periods after the administration of T.E.D. The response in renal hemodynamics and sodium excretion to this drug was variable both in magnitude and duration. The elevated filtration rate was not sustained throughout the experiment in a single case and was increased for one period only

TABLE III
(Experiment IV)
The effect of theophylline ethylene diamine on renal function in patients with cardiac failure

Case No.		Control periods				Periods after administration of theophylline ethylene diamine				
		1	2	3	Average	4*	5	6	7	8
1	Glomerular Filtration Rate cc./min./1.73 sq.m.	92.4	99.8	108.1	100.1	239.1	94.6	102.4	89.5	106.0
2		98.5	97.2	98.4	98.0	143.1	113.8	136.3	107.8	85.8
3		131.2	140.0	132.3	134.5	143.0	125.6	121.7	119.2	123.7
4		75.4	70.8	58.9	68.4	69.8	75.2	74.5	—	—
5		59.9	58.6	57.1	58.5	72.1	54.4	61.5	60.5	65.5
6		27.6	30.0	25.8	27.8	31.6	23.2	24.7	25.3	26.0
1	Effective Renal Plasma Flow cc./min./1.73 sq.m.	154.0	186.1	192.0	177.4	388.0	185.0	194.7	155.1	195.2
2		217.6	214.0	209.2	213.6	359.1	266.6	283.7	256.0	208.4
3		272.6	295.2	275.7	281.6	382.0	329.0	271.3	307.8	324.0
4		199.0	183.4	154.7	179.3	185.3	164.2	153.8	—	—
5		198.6	170.1	167.9	178.8	218.1	197.1	201.8	202.5	193.9
6		137.9	128.8	131.4	132.7	163.0	130.3	131.9	127.9	141.3
1	Filtration Fraction per cent	60.0	53.6	56.3	56.6	61.6	51.1	52.6	57.7	54.3
2		45.3	45.4	47.0	45.9	39.8	42.7	48.0	42.1	41.2
3		48.1	47.4	48.0	47.8	37.4	38.2	44.9	38.7	38.2
4		37.9	38.6	38.0	38.2	37.7	45.8	48.4	—	—
5		30.1	34.4	34.0	32.8	33.0	27.6	30.5	29.9	33.8
6		20.0	23.3	19.7	21.0	19.4	17.8	18.5	19.8	18.4
1	Urine Flow cc./min./sq.m.	2.45	1.59	1.49	1.84	6.63	4.73	3.54	3.66	3.06
2		—	.34	.28	.21	1.80	1.65	2.72	1.74	2.01
3		—	1.22	.51	.58	5.99	7.65	5.09	4.75	5.23
4		1.74	1.60	1.16	1.50	2.22	2.46	1.89	—	—
5		5.42	6.68	5.42	5.84	11.12	5.60	4.12	5.36	4.82
6		1.61	1.47	2.30	1.79	3.17	2.24	1.70	.90	.94
1	Sodium Clearance cc./min.	.50	.53	.58	.53	4.80	3.06	2.81	2.52	2.77
2		.14	.14	.14	.14	2.82	3.29	3.55	3.39	2.90
3		.41	.41	.37	.40	7.36	9.34	6.65	6.45	6.68
4		1.53	1.28	1.16	1.32	3.78	4.14	4.10	—	—
5		4.10	5.13	4.69	4.64	6.78	4.90	4.85	4.59	5.65
6		2.69	2.67	2.59	2.65	3.82	2.98	2.30	2.17	1.98
1	Concentration of Sodium in Urine m.eq./cc.	.019	.031	.036	.028	.066	.059	.073	.063	.083
2		—	.036	.043	.029	.138	.176	.113	.172	.127
3		—	.031	.068	.033	.115	.114	.122	.127	.120
4		.063	.058	.072	.064	.121	.120	.154	—	—
5		.064	.065	.073	.067	.052	.074	.099	.072	.099
6		.132	.145	.089	.123	.095	.106	.108	.193	.167

* Injection of 0.48 gm. of drug during the first five minutes of the fourth clearance period

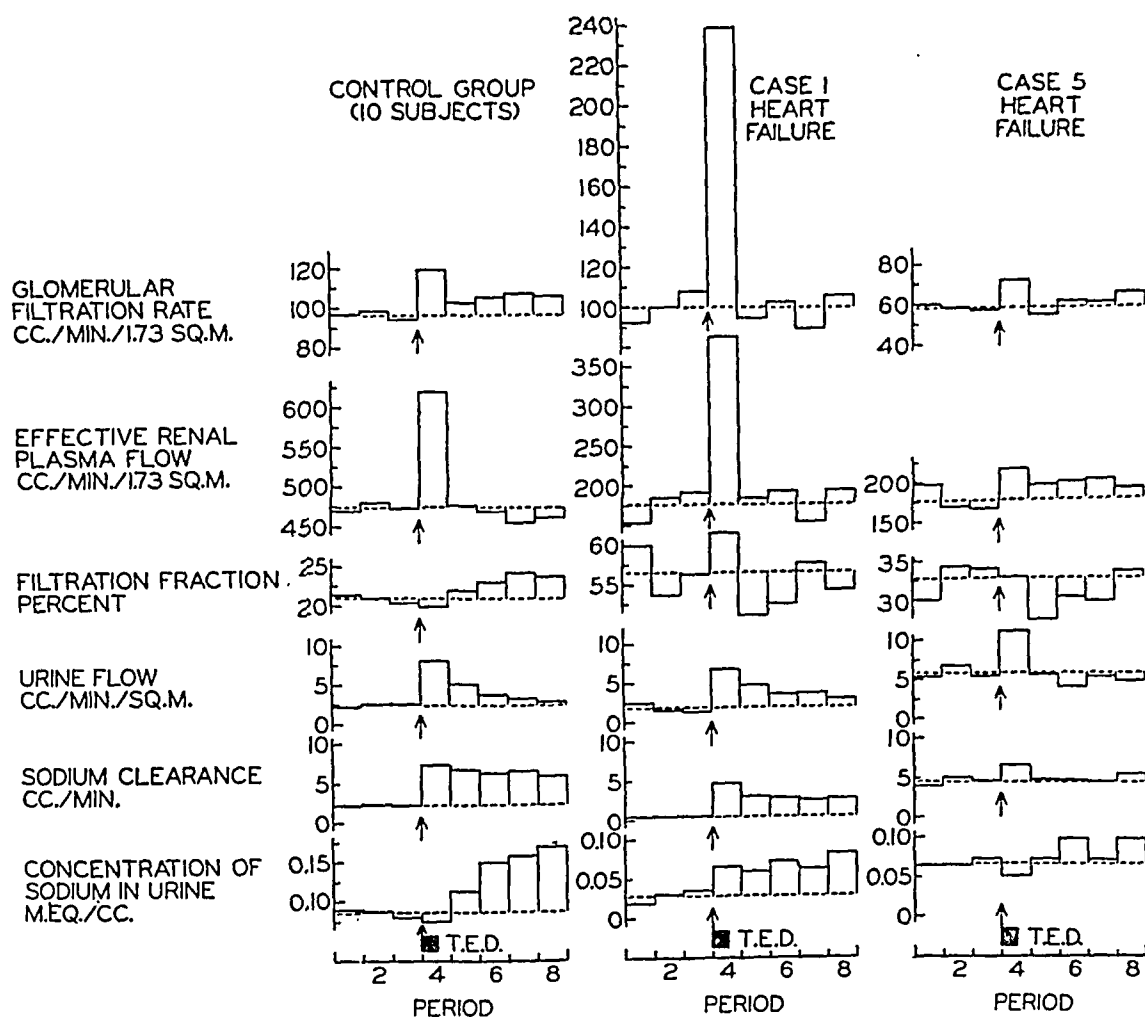


FIG. 1. THE EFFECT OF THEOPHYLLINE ETHYLENE DIAMINE ON RENAL FUNCTION IN A GROUP OF 10 CONTROL SUBJECTS (EXPERIMENT III) AND IN TWO PATIENTS WITH CONGESTIVE HEART FAILURE (EXPERIMENT IV)

Case 1 with heart failure showed a normal filtration rate but sodium clearance was low during the control periods; a maximal response to T.E.D. occurred. Case 5 with heart failure showed a low filtration rate but an elevated sodium clearance was obtained during the control periods; a minimal response to T.E.D. occurred. The arrows indicate the time of the beginning of the injection of T.E.D. The dotted line represents the mean value for the three periods before T.E.D.

in two of the three cases in which a significant response occurred. The rise in effective renal plasma flow was also transitory. The increase in sodium and water excretion varied from a moderate elevation in Case 4 to a marked rise in Cases 1 and 3. As in the control study (Experiment III) the elevation in sodium clearance was sustained for five clearance periods in Cases 1-4 but was increased during period 4 only in Cases 5 and 6. The water diuresis was maintained throughout the experiment in Cases 1-4 as compared with the more transitory diuresis in the controls. The concentration of sodium in the urine increased after T.E.D. in Cases 1-4 and remained at the elevated level in accordance with the sustained in-

crease in both urine flow and sodium clearance. In Cases 5 and 6 the changes in the concentration of sodium in the urine were insignificant.

DISCUSSION

Mechanism of xanthine diuresis

Studies of the diuretic action of xanthines have yielded conflicting findings both in lower animals and man. As a result, two principal theories have been formulated to explain the mechanism of xanthine diuresis. First, it has been proposed that diuresis is primarily the result of augmented glomerular filtration rate secondary to an increase in renal blood flow. Secondly, investigators have

presented evidence that xanthines exert their action on the renal tubules to decrease reabsorption of water and electrolytes.

Effect of xanthines on renal hemodynamics. In lower animals, some xanthines increase filtration rate, while others do not. For instance, Schmitz (18) reported that euphyllin consistently increased glomerular filtration rate in dogs while others (19-21) found renal hemodynamics unaltered significantly after the injection of theophylline in rabbits and dogs.

In man the results have been more uniform. With the exception of an early report by Blumgart and associates (22), studies (11, 23-27) have demonstrated repeatedly an alteration in renal hemodynamics. However, there is considerable variation in the magnitude and duration of the response.

In the present study, a sustained increase in glomerular filtration rate occurred consistently in the 25 control subjects while the response in patients with congestive heart failure was variable. In normal subjects, Howarth and associates (28) reported a 35% increase in cardiac output which lasted for 10-15 minutes after the intravenous administration of 0.48 gm. of T.E.D. Consequently, the sustained elevation in glomerular filtration in control subjects may be explained on the basis of renal arteriolar dilatation, while the marked initial response in Experiments II and III may be the result of a transitory elevation in cardiac output superimposed upon alterations in the renal arterioles. The more transitory response in filtration rate in patients with cardiac failure suggests that T.E.D. has less effect upon the renal arterioles in these patients than in controls. Furthermore, the marked immediate elevation and transitory response in glomerular filtration in some cases with cardiac failure (Cases 1 and 2) may be primarily the result of increased cardiac output rather than renal arteriolar dilatation.

Since most of the control values of renal plasma flow in elderly subjects are low when compared with the values reported for the middle and young age groups (16, 29), the question arises as to the completeness of renal diodrast extraction. From the available data this question cannot be answered and renal plasma flow has been referred to as "ef-

fective renal plasma flow." An increase in diodrast clearance may be indicative of an increase in renal plasma flow after the administration of T.E.D. unless the renal diodrast extraction ratio is increased by improving the cellular function of the tubules so that they extract a higher percentage of diodrast. The changes in effective renal plasma flow may then be attributed to possible alterations in (1) cardiac output, (2) afferent and efferent arteriolar size, and (3) diodrast extraction.

Effect of T.E.D. on tubular reabsorption. Consideration of the temporal relationship of the response in filtration rate to the rise in sodium clearance reveals that both were sustained for 50-60 min. in the control subjects. However, Cases 1-4 with congestive heart failure showed a very transitory or no increase in filtration rate while sodium clearance was maintained at an elevated level throughout the experiment. This finding that T.E.D. decreases sodium reabsorption was presented in a preliminary report by Davis and Shock (27) and confirmed by Sinclair-Smith and associates (11).

Evidence that T.E.D. results in decreased water reabsorption is suggested from the relationship of the level of filtration rate to urine flow in the control subjects. The elevated filtration rate was maintained for five periods after T.E.D. but urine flow returned to the control level during periods 7 and 8 (Figure 1). Since the tubules possess the capacity to reabsorb the increased load of water imposed by the elevated filtration rate during periods 7 and 8, the increase in urine flow during periods 4-6 may be explained on the basis of T.E.D. inhibiting water reabsorption.⁴ Furthermore, urine flow was maintained at an elevated level in cases of cardiac failure in whom the response in filtration rate was transitory. These findings indicate that the sustained diuretic effect of T.E.D. is primarily the result of its action (direct or indirect through the posterior pituitary) on renal tubules. The lack of an elevated urine flow in the presence of a high sodium clearance (periods 7-8) indicates that this level of sodium excretion was insufficient to effect an appreciable osmotic diuresis.

⁴ This assumes that T_m for water remains constant for periods 4-8. For a discussion of T_m for water see Wesson, Anslow, and Smith (12).

Renal Function in Congestive Heart Failure

Clearance determinations during the control periods revealed a normal glomerular filtration rate in the presence of a low sodium clearance in Cases 1-4. In Case 4, the glomerular filtration rate of 68.4 cc./min./1.73 sq.m. may seem abnormally low for even an 82-year-old man, but this is the same order of magnitude as reported by Davies and Shock (29) for men 80-90 years of age who showed no evidence of cardiac or renal disease from history, physical examination and urinalysis. Cases 5 and 6 showed a low filtration rate but sodium clearance was higher than the mean value for the control group. These findings of the lack of correlation between the level of filtration rate and sodium clearance coincide with the results of other studies (10, 11, 30) and indicate that filtration rate is not the primary mechanism for salt retention in cardiac failure.

The incidence of a normal filtration rate in one-half to two-thirds of the patients with cardiac failure in this series may be unusually high. However, examination of the results of other investigators indicates that a normal filtration rate is not uncommon (4, 10, 11, 30). Farnsworth and Krakusin (30) reported one of two cases and Sinclair-Smith and associates (11) found one of three cases who consistently showed a normal filtration rate during long term electrolyte balance and renal function studies. Briggs and coworkers (10) noted a glomerular filtration rate above 105 cc./min./1.73 sq.m. in three of 16 cases. When account is taken of the decline in filtration rate with age (29), the per cent of reported cases with a normal glomerular filtration rate in a large series such as Merrill's becomes significant.

The two cases (5 and 6) who showed an elevated sodium clearance are unusual. Certain data suggest that they had intrinsic renal tubular disease before the onset of congestive heart failure. After the state of cardiac compensation had improved, concentration tests revealed a low maximal specific gravity of the urine (1.015 and 1.018). In both cases an advanced degree of generalized arteriosclerosis was present. Although it is not possible to predict the extent of renal involvement from the degree of arteriosclerosis in the peripheral vessels, arteriosclerosis would offer a likely explanation for such renal involvement. It seems unlikely that an elevated

sodium intake was present in these two patients since they were on a diet similar to the control subjects and two of the patients with a low sodium clearance. Furthermore, the elevated sodium clearance was not the result of improvement during diuresis as described in some cases of Threefoot, Burch, and Reaser (31) because these patients were becoming progressively more decompensated before the experiment. It may be of some significance that the control subjects and patients in cardiac failure received normal saline in both the priming and sustaining solutions. Since they received approximately 3 cc. of normal saline per minute, .426 m.eq./min. of sodium chloride was infused. This value is of the same order of magnitude as the quantity of sodium excreted (.639 and .366 m.eq./min. in Cases 5 and 6 respectively).

The experimental alteration of renal hemodynamics in heart failure by T.E.D. proved to be of little importance in studying the mechanism of salt and water retention. Since T.E.D. decreases tubular reabsorption of sodium, it tends to obscure any close correlation which might exist between sodium excretion and the tubular load of sodium. It should be pointed out, however, that an elevation in glomerular filtration rate was frequently unaccompanied by a corresponding increase in sodium clearance. In other words, the tubules were capable of reabsorbing approximately two and one-half the tubular load of sodium during period 4 as compared with periods 5-8 (see Case 1).

Since the evidence presented in this study offers no support for the hypothesis that salt retention is the result of a low filtration rate, the theory that renal impairment in cardiac failure is related to tubular dysfunction should be investigated more thoroughly. A low filtration rate may lead to salt retention by increasing tubular reabsorption of sodium or by some other mechanism in some patients but in other cases the kidney retains sodium in the presence of a normal glomerular filtration rate. The exact mechanism responsible for the accumulation of salt and water in heart failure awaits a more complete understanding of the tubular reabsorptive mechanism of sodium in normal individuals. Investigation of the role of extra-renal factors such as extracellular fluid volume, adrenal cortical hormones, and the secretion of the posterior pituitary may help to clarify this complicated problem.

SUMMARY

The effect of theophylline ethylene diamine on renal function was studied in 25 control subjects and in six patients with congestive heart failure. Venous pressure was measured in 10 of the control subjects and in four of the patients with cardiac failure.

In the control subjects, an increase in filtration rate was sustained for 50–60 minutes. The response in effective renal plasma flow varied from a transitory elevation lasting 10–12 minutes to an elevation followed by a gradual return to the control level or below. Filtration fraction was usually unchanged during the first two periods after T.E.D. but was elevated during the remaining three clearance periods. Urine flow trebled immediately after the injection of T.E.D. and gradually returned to a value slightly above the control level during the next 50 or 60 minutes. Sodium clearance increased threefold and remained elevated throughout the test. The concentration of sodium in the urine was unaltered immediately after the injection of T.E.D. but gradually increased to a level double the control. The mean decrease in venous pressure was 17 per cent of the control value.

During the three control periods the patients in congestive heart failure showed a filtration rate within normal limits in Cases 1–4 while effective renal plasma flow was consistently reduced. Urine flow was below the level for the control subjects in these four patients. In Cases 5 and 6, both filtration rate and effective renal plasma flow were greatly diminished. Urine flow was elevated in Case 5 but depressed in Case 6. Filtration fraction was elevated in all but one of the six patients. The concentration of sodium in the urine was decreased below the value for the control group in all but one case.

The response in renal hemodynamics to T.E.D. in patients with cardiac failure was variable. Filtration rate and effective renal plasma flow failed to increase at all in some patients and increased over 100 per cent in others. In Cases 1–4 urine flow and sodium clearance varied from a slight to a marked response. In Cases 5 and 6 urine flow and sodium clearance increased slightly only immediately after the injection of T.E.D. The concentration of sodium in the urine increased

immediately after T.E.D. in Cases 1–4 but remained unchanged in Cases 5 and 6 except for a slight increase during the last clearance periods. The response in venous pressure to T.E.D. varied with the height of the initial level; in patients with a control tension of approximately 10.0 cm. of saline the response was similar to that observed in control subjects while one patient with a high venous tension before T.E.D., showed a marked fall.

CONCLUSIONS

The mechanism of the diuretic action of T.E.D. in man is apparently two fold. An increase in the excretion of sodium and water is associated with (1) an elevation in glomerular filtration rate, and (2) a decrease in renal tubular reabsorption of sodium and water. Although a quantitative evaluation of the relative importance of each of these factors cannot be made from the available data, it seems that a decrease in tubular reabsorption contributes more to the sustained diuresis than augmented filtration rate.

The evidence from this study offers no support for the concept that salt and water retention by the kidney in patients with congestive heart failure is the result of low filtration rate. Consequently, it appears that the mechanism of renal impairment in cardiac failure is to be sought in a study of the tubular reabsorptive mechanism of sodium.

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ELECTROPHORETIC STUDIES OF THE SERUM PROTEINS IN BILIARY CIRRHOSIS

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Although the serum proteins in diseases of the liver have been studied by the electrophoretic method by many investigators, few references have been made to biliary cirrhosis (1-3). Since a systematic study has not as yet appeared, the present investigation of ten cases of obstructive biliary cirrhosis was undertaken.

METHODS

Electrophoretic runs were performed with standard apparatus and technique, the experimental details having been reported previously (4). The buffer was sodium diethylbarbiturate (veronal) of pH 8.6 and ionic strength 0.1.

In the estimation of the fractions as per cent of the serum proteins both ascending and descending patterns were used, the areas being divided by ordinates from the nadirs between peaks to the base line. The quantities of the fractions as grams per cent represent the products (4, 5) of the percentages and the total proteins from the conventional Kjeldahl method, using a nitrogen factor of 6.25. Such a method of computation involves the assumptions that the specific refractive increments and the nitrogen contents of the various protein fractions are identical. These assumptions which have been generally employed as first approximations must be modified somewhat in view of the quantitative studies of Armstrong and colleagues (6, 7). In practice the errors thus introduced appear ordinarily no greater than others inherent in the electrophoretic method. However, where there is considerable increase in the lipids associated with the beta-globulin complex, the nitrogen content of this fraction is low in comparison with its refractive increment which determines the size of the peak in the electrophoretic diagram. In such a circumstance, which occurred in some of the cases of the present series, the errors in computing the absolute concentrations of the protein fractions may become appreciable. On the other hand, the accuracy of the values for the relative proportions of the fractions (as per cent of the serum proteins) should not be seriously affected.

The tests of hepatic function were performed by the usual methods as previously described (4), alkaline phosphatase being reported in Bodansky units. Cholesterol

and cholesterol esters were determined by the method of Schoenheimer and Sperry (8).

Ether extraction of the serum was carried out in one case by performing three extractions with redistilled diethyl ether, shaking thoroughly before each separation. The procedure was carried out in a cold room at 4° C. The ether layer acquired the coloration of the serum, the hue being progressively less intense on successive extractions so that in the third, the ether layer was almost colorless.

CASE MATERIAL

The present study comprises ten cases of obstructive biliary cirrhosis. In all patients cholelithiasis had been present and cholecystectomies had been performed. All had been intensely jaundiced or were still jaundiced at the time of study. In six cases (L. S., L. H., K. V., M. G., W. H., and T. O.) development of biliary cirrhosis followed cholecystectomy, and four of these patients (L. S., L. H., W. H., and T. O.) had subsequent laparotomies in the attempt to repair a severely stenosed common duct. In four cases (F. F., M. E., M. F., and C. M.) biliary cirrhosis was already present at the time of cholecystectomy and hence could not be attributed to surgical trauma to the common duct.

The diagnosis was confirmed histologically from tissue obtained at laparotomy or by needle (Vim Silverman) biopsy of the liver in eight of the ten cases. The other two (M. G. and W. H.) were clinically typical cases, and in one of them (W. H.) an enlarged bile-stained liver was observed at three laparotomies for repair of the severely stenosed common duct.

The series has been confined to cases of biliary cirrhosis of the obstructive type, other etiologies such as parasitic infestation, etc. being excluded. No cases of essential xanthomatosis were studied.

RESULTS

Typical electrophoretic patterns are illustrated in Figure 1 and the data on the serum proteins as well as the tests of hepatic function are listed in Table I, roughly in order of increasing deviation from normal serum protein composition. The normal controls have been reported previously (4).

The electrophoretic analyses revealed diminutions of the serum albumin fractions in all cases

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TABLE I

Bromsulfalein tests on the two patients with normal serum bilirubin values, W. H. and M. F., revealed 3 per cent and 48 per cent retention respectively

BILIARY CIRRHOSIS

Patient	Electrophoretic data											Howe sodium sulfate				Tests of hepatic function						
	per cent					grams per cent					A/G	A/G	grams per cent			bilirub		ceph	thym	alk phos	chol	chol est
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ			Alb	Glob	Tot	dir	tot					
W.H.	54.6	5.0	9.0	17.7	13.7	3.78	0.35	0.62	1.22	0.95	1.20	2.04	4.64	2.28	6.92	0.5	0.9	neg.	2.7	2.3	270*	205*
M.F.	44.8	5.4	10.2	16.3	23.3	3.40	0.41	0.78	1.24	1.77	0.81	1.33	4.34	3.26	7.60	0.6	0.9	2+	6.9	35.1*	230	172
T.O.	42.1	6.6	13.3	27.9	10.1	2.69	0.42	0.85	1.79	0.65	0.73	1.91	4.20	2.20	6.40	13.4	18.2	neg.	1.1	—	355	155
M.G.	47.9	4.1	9.2	20.3	18.5	3.44	0.29	0.66	1.46	1.33	0.92	1.37	4.15	3.03	7.18	1.0	1.5	3+	6.2	6.8	255	188
C.M.	40.8	7.2	16.1	15.7	20.2	2.49	0.44	0.98	0.96	1.23	0.69	1.44	3.60	2.50	6.10	1.8	2.9	3+	11.8	31.5	232	95
F.F.	37.8	8.8	16.3	21.0	16.1	2.47	0.57	1.06	1.37	1.05	0.61	1.19	3.54	2.98	6.52	3.5	4.6	1+	9.2	12.3*	268	165
K.V.	36.9	6.9	12.1	23.7	20.4	2.03	0.38	0.67	1.30	1.12	0.59	1.08	2.86	2.64	5.50	12.0	17.2	3+	12.7	10.4*	400*	60*
M.E.	39.0	6.6	9.4	18.8	26.2	3.12	0.53	0.75	1.50	2.10	0.64	0.98	3.95	4.05	8.00	0.9	1.7	4+	18.8	41.7	350	245
L.S.	29.6	5.3	9.3	25.6	30.2	2.53	0.45	0.79	2.19	2.58	0.42	0.72	3.58	4.96	8.54	2.3	3.2	3+	15.0	49.9	470	125
L.H.	32.4	6.0	10.9	32.7	18.0	2.92	0.54	0.99	2.95	1.63	0.48	0.80	4.00	5.03	9.03	12.1	16.3	4+	20.0	63.4	408	145
AVERAGE	40.6	6.2	11.6	22.0	19.6	2.89	0.44	0.81	1.60	1.44	0.71	1.29	3.89	3.29	7.18	* starred values not simultaneous with other data						
Standard Deviation	6.9	1.3	4.0	5.2	5.6	0.52	0.08	0.21	0.55	0.55	0.21	0.41	0.48	0.99	1.06							

NORMAL SERA
(10 volunteer subjects with normal liver function tests)

AVERAGE	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93	1.52	2.31	4.89	2.12	7.01
Standard Deviation	2.8	0.8	1.5	1.2	1.7	0.29	0.06	0.10	0.08	0.13	0.18	0.34	0.30	0.24	0.30

even when normal by the Howe method. The gamma-globulins were elevated in eight of the ten cases. The beta-globulins were elevated in all cases, with some strikingly high values, a finding which will be further discussed. Significant increases of the alpha-one- and alpha-two-globulins occurred less frequently.

As shown in Table I, hypercholesterolemia with decreased cholesterol esters was observed although not a constant finding. Several unusually high alkaline phosphatase activities occurred, and all but two values were above normal. Cephalin cholesterol flocculation and thymol turbidity tests were positive in all but four and two instances respectively.

On the basis of their clinical status at the time of the study, the cases fell into three groups which bore some relationship to the electrophoretic data.

In the first group of two cases (W. H. and M. F.) the pathologic process was apparently quiescent and the serum bilirubin values were normal. W. H. had been entirely asymptomatic for five

months with normal tests of hepatic function including bromsulfalein retention of 3 per cent; the electrophoretic analysis showed slightly reduced albumin, elevated beta-globulin, and normal gamma-globulin. M. F. with minimal symptoms (pruritus, fatigability) for 11 months had a high alkaline phosphatase (35.1 units) and bromsulfalein retention of 48 per cent, despite normal serum bilirubin; the electrophoretic analysis revealed moderately diminished albumin and elevated beta- and gamma-globulins.

The second group of five patients (T. O., M. G., C. M., F. F., and K. V.) had active ascending cholangitis at the time of the study with varying degrees of elevation of the serum bilirubin. The electrophoretic patterns showed greater deviation from the normal than the quiescent group with, however, considerable variation. The albumin values of all but one (M. G.) were below 3 grams per cent. T. O. exhibited a strikingly high beta-globulin peak without gamma-globulin increase. M. G. and K. V. showed increased beta- and

gamma-globulins. C. M. and F. F. had some elevation of all the globulin fractions, the former with high gamma-globulin, the latter with high beta-globulin.

The third group of three patients (M. E., L. S., and L. H.) exhibited secondary xanthomatosis (palmar and palpebral) after long histories of obstructive jaundice. M. E. was studied three years after the onset of episodes of chills, fever and jaundice, and five months after cholecystectomy which had been followed by decline of the serum bilirubin to a value only slightly above the normal range. The electrophoretic analysis showed marked abnormality with diminished albumin and elevated beta- and gamma-globulins, but the pattern was far less striking than those of L. S. and L. H., the most bizarre in the series. These patients had almost complete occlusion of the common duct due to stricture following cholecystectomy five years ago in each case, and attempts at

operative repair had been futile. The onset of progressive secondary xanthomatosis occurred during periods of clinical observation long after histologic diagnoses of obstructive biliary cirrhosis had been made. The electrophoretic patterns showed spectacular beta-globulin elevations. Both had marked diminutions of the albumin fractions. The beta-globulin fraction of L. S. was exceeded by the even higher gamma globulin, which was the highest in all the cases studied. The beta-globulin of L. H. was over three times the normal in grams per cent and exceeded each of the other fractions of the serum.

Changes of electrophoretic patterns during the course of the disease were followed in three illustrative cases (K. V., L. S., and L. H.). All were given a high protein, high carbohydrate and high caloric diet containing 120 to 150 grams of protein, 350 to 450 grams of carbohydrate, a total of 3,000 to 3,500 calories, supplemented by 4 to

ELECTROPHORETIC PATTERNS OF SERUM PROTEINS IN BILIARY CIRRHOSIS

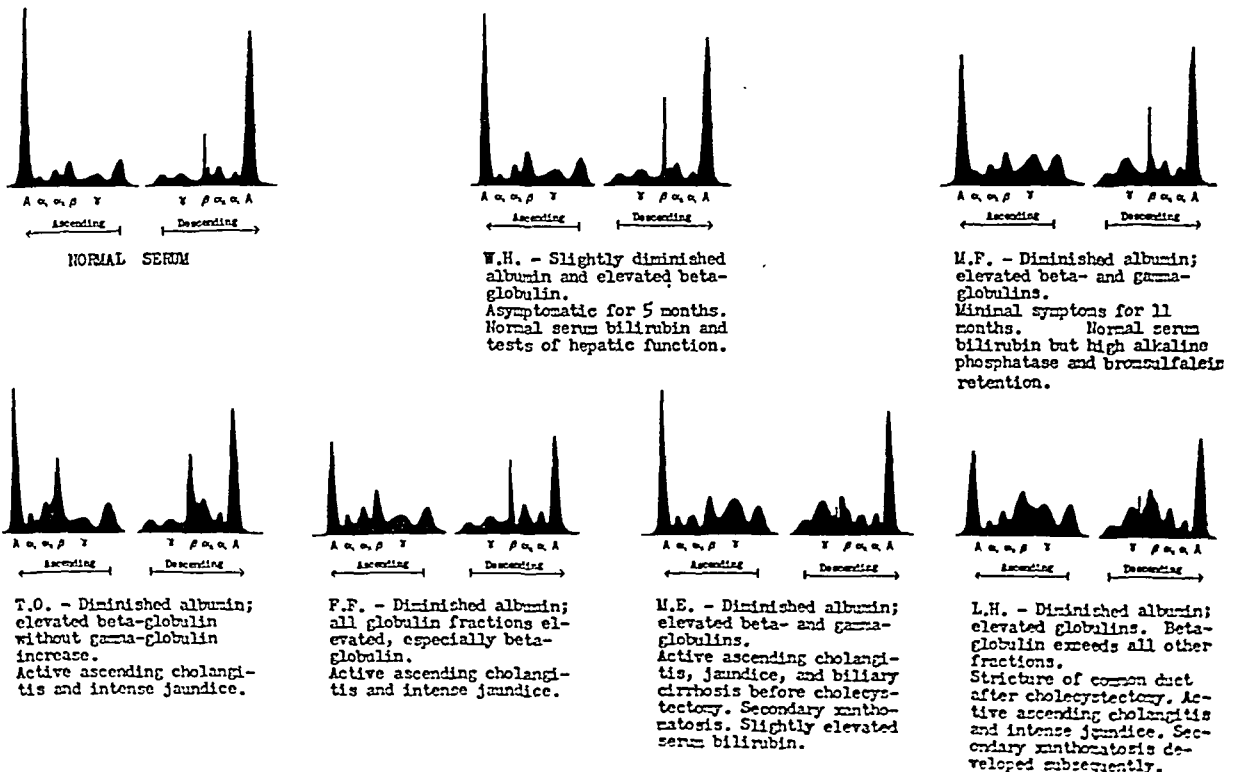


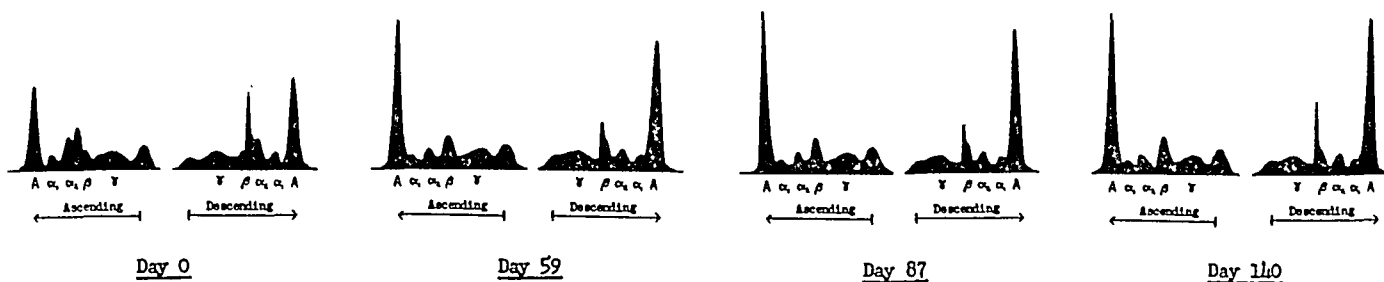
FIG. 1

The albumin peaks are indicated by "A," and the globulins by their respective Greek letter prefixes. The unlabelled peaks are the stationary anomalous boundaries due to gradients of buffer salt.

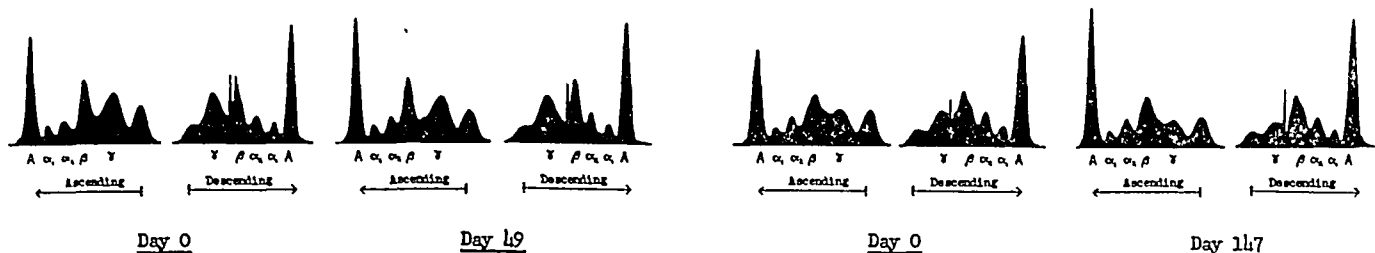
TABLE II
COURSE OF BILIARY CIRRHOSIS

	Electrophoretic data											Howe sodium sulfato				Tests of hepatic function							
	per cent					grams per cent					A/G	grams per cent			bilirub		ceph	thym	alk phos	chol	chol est		
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ		A/G	Alb	Glob	Tot	dir						tot	
Sera of K.V.																							
Day 0	36.9	6.9	12.1	23.7	20.4	2.03	0.38	0.67	1.30	1.12	0.59	1.08	2.86	2.64	5.50	12.0	17.2	3+	12.7	10.4	400*	60*	
Day 59	49.3	5.0	9.4	17.4	18.9	3.44	0.35	0.66	1.22	1.32	0.97	1.24	3.87	3.12	6.99	0.8	1.3	neg.	10.9	8.8	290	200	
Day 87	51.4	5.3	9.4	16.0	17.9	3.62	0.37	0.66	1.13	1.26	1.06	0.94	3.41	3.63	7.04	0.3	0.5	neg.	8.4	---	228	165	
Day 140	53.1	4.8	9.0	17.2	15.9	3.61	0.33	0.61	1.17	1.08	1.13	1.56	4.14	2.66	6.80	0.1	0.3	1+	6.5	5.1	293	210	
* starred values not simultaneous with other data																							
Sera of L.S.																							
Day 0	29.6	5.3	9.3	25.6	30.2	2.53	0.45	0.79	2.19	2.58	0.42	0.72	3.58	4.96	8.54	2.3	3.2	3+	15.0	49.9	470	125	
Day 49	31.4	5.1	8.9	23.8	30.8	2.80	0.45	0.79	2.12	2.74	0.46	0.67	3.56	5.34	8.90	7.2	10.6	3+	20.0	14.8	570	115	
Sera of L.H.																							
Day 0	32.4	6.0	10.9	32.7	18.0	2.92	0.54	0.99	2.95	1.63	0.48	0.80	4.00	5.03	9.03	12.1	16.3	4+	20.0	63.4	408	145	
Day 147	40.1	4.9	11.0	31.1	12.9	3.23	0.39	0.89	2.50	1.04	0.67	1.00	4.02	4.03	8.05	21.6	28.0	1+	6.8	48.0	450	100	

COURSE OF BILIARY CIRRHOSIS



K.V. - Cholecystectomy with rubber abdominal drain three months previously followed by intense jaundice and ascending cholangitis. Insertion of T-tube into slightly dilated common duct. Subsidence of jaundice and all symptoms. Alteration of electrophoretic pattern in the direction of normal by Day 59; little change thereafter.



L.S. - Irreparable stricture of common duct after cholecystectomy five years ago. Progressive development of secondary xanthomatosis. No significant change in electrophoretic pattern.

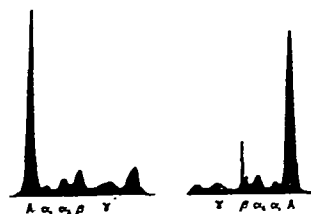
L.H. - Irreparable stricture of common duct after cholecystectomy five years ago. Progressive development of secondary xanthomatosis. Little change in electrophoretic pattern.

FIG. 2

NORMAL SERUM

Figures are averages of ten volunteer subjects with normal tests of hepatic function.

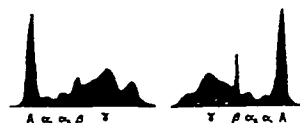
	Alb	α_1	α_2	β	γ	A/G
per cent	60.3	4.0	9.7	12.8	13.2	1.52
grams per cent	4.23	0.28	0.68	0.89	0.93	
Howe sod. sulfate	4.89	Glob-2.12	Tot-7.01			2.31



PORTAL CIRRHOSIS

Intense jaundice, massive ascites and edema in a patient with advanced portal cirrhosis (needle biopsy).

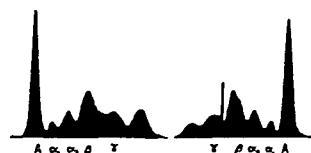
	Alb	α_1	α_2	β	γ	A/G
per cent	38.1	4.1	6.4	12.6	38.8	0.62
grams per cent	2.56	0.28	0.43	0.85	2.61	
Howe sod. sulfate	2.99	Glob-3.74	Tot-6.73			0.80



BILIARY CIRRHOSIS

L.H. - Irreparable stricture of common duct after cholecystectomy 5 years ago. Intense jaundice. Secondary xanthomatosis.

	Alb	α_1	α_2	β	γ	A/G
per cent	40.1	4.9	11.0	31.1	12.9	0.67
grams per cent	3.23	0.39	0.89	2.50	1.04	
Howe sod. sulfate	4.02	Glob-4.03	Tot-8.05			1.00



BILIARY CIRRHOSIS

(ETHER-EXTRACTED SERUM)

Same serum as above after three extractions with redistilled diethyl ether.

	Alb	α_1	α_2	β	γ	A/G
per cent	44.8	5.2	13.2	14.5	22.3	0.81

Beta-globulin peak reduced to approximately one third of original area.

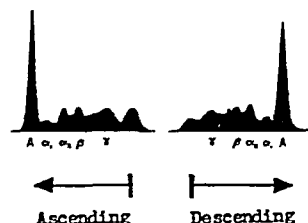


FIG. 3

6 grams of choline chloride daily. During periods of acute ascending cholangitis sulfadiazine and/or penicillin was administered.

The electrophoretic patterns and data of these three patients are shown in Figure 2 and Table II.

K. V. had had a cholecystectomy three months previously with a rubber abdominal drain left in place, followed by intense jaundice and ascending cholangitis. After laparotomy with insertion of a T-tube into the slightly dilated common duct (ten days prior to Day 0) the jaundice and all symptoms subsided. The serial electrophoretic patterns showed evolution in the direction of normal with definite rise of the diminished albumin fraction

and some decline of the elevated beta- and gamma-globulins. The most pronounced change had occurred by Day 59, the pattern showing only minor alteration thereafter. During the period of study there was also change of the tests of hepatic function toward normal.

L. S. and L. H. with irreversible obstructive jaundice (severe stricture of the common duct) and secondary xanthomatosis manifested no fundamental change in clinical status during observation, although recurring episodes of acute ascending cholangitis with chills and fever were prevented by sulfadiazine (2 to 6 grams daily). The electrophoretic pattern of L. S. showed no

significant change after 49 days. After 147 days, L. H. exhibited some albumin rise and decline of the elevated gamma-globulin, while the pattern retained the same bizarre general configuration with the striking beta-globulin peak.

In contrast with these findings, portal cirrhosis has not shown marked elevation of the beta-globulin fraction as a constant or conspicuous feature. Comparison of the electrophoretic patterns of deeply jaundiced cases of biliary and portal cirrhosis is illustrated in Figure 3 where the difference between the beta-globulin peaks is striking.

Ether extraction of the serum of the case of biliary cirrhosis resulted in pronounced reduction of the beta-globulin peak to approximately one-third its original area, the conspicuous beta-globulin disturbance (spike) of the descending pattern being likewise markedly diminished. This experiment indicates the important contribution of lipo-protein to the high beta-globulin.

DISCUSSION

The ten cases of biliary cirrhosis had diminished albumin values in all instances, even when normal by the Howe method. The gamma-globulins were elevated in eight of the ten cases. The most characteristic and conspicuous feature, however, was elevation of the beta-globulins, observed in all cases and reaching spectacular levels in two cases with secondary xanthomatosis (L. S. and L. H.). These two cases with almost complete occlusion of the common duct due to post-operative stricture of five years' duration exhibited the most abnormal electrophoretic patterns of the series, one of them (L. H.) showing a beta-globulin exceeding any of the other fractions. The electrophoretic pattern nearest normal occurred in a patient (W. H.) who had been entirely asymptomatic for five months and had normal tests of hepatic function. This pattern revealed slightly diminished albumin, elevated beta-globulin and normal gamma-globulin.

The data in these cases of biliary cirrhosis contrasted with the findings in a previously reported series of cases of portal cirrhosis (4) in which elevation of the beta-globulin fraction was a less constant and less conspicuous feature, strikingly tall peaks never having been observed even when the beta-globulin was high. Moreover, in the cases of portal cirrhosis beta-globulin increases did

not occur without marked elevation of gamma-globulin.

It is of interest to compare these findings with the isolated cases previously reported. The patient with "xanthomatosis and biliary cirrhosis" of Gray and Barron (1) exhibited a serum protein composition strikingly similar to our cases with secondary xanthomatosis. The two cases of Thorn, Armstrong and Davenport (2) had major concomitant disease: in one, the biliary cirrhosis was secondary to bile duct carcinoma extensively infiltrating the liver, and in the other biliary cirrhosis was superimposed on old portal cirrhosis, the electrophoretic analyses showing slightly elevated and normal beta-globulins respectively. The patients evidently do not bear close comparison with the present series. A single case listed among the data of Cohen and Thompson (3) had only slight elevation of the beta-globulin but a very high gamma-globulin.

The experiment showing reduction of the greatly increased beta-globulin peak to approximately one-third its original area by ether extraction was interpreted as demonstrating the important contribution of lipo-protein to this pathologically elevated fraction. The same phenomenon has been observed by Longsworth and colleagues in sera of patients with nephrosis (9) and obstructive jaundice (10), the one difference being that the descending beta-globulin disturbance (spike) was essentially unchanged, whereas in the present experiment it was markedly diminished after ether extraction. Zeldis, Alling and co-workers (11) reported somewhat similar results but with complete abolition of the beta-globulin disturbance employing an alcohol-ether extraction that probably caused partial denaturation of the proteins. These findings were obtained in patients with nephrosis, "obstructive jaundice with cholangitis," and other diseases. Unpublished work by Lever (12) corroborates and extends the present findings with cold ether extraction.

Longsworth (9) has discussed the distinction between the lipids presumably present as suspended globules coated with beta-globulin, notably in lipemic (milky) sera which can be clarified by high speed centrifugation, and on the other hand the lipo-proteins in clear solution, which in pathologic sera (such as the present series) may be extracted by cold ether with reduction of the

abnormally high beta-globulin peak. It should be noted that the alpha- and beta-lipo-proteins of normal sera have the solubility properties of proteins (13, 14) and are not ether-extractable to any extent (9, 14). The present elevated beta-lipo-protein may differ essentially from the normal. An alternative suggestion is the possible effect of bile salts in the serum on the stability of the lipo-protein complex (15, 16).

Kunkel and Ahrens (17, 18) have emphasized the role of the phospholipid content of the lipo-proteins. The phospholipids were found to be relatively low in the lipemic (milky) sera of patients with nephrosis, but high in the transparent high-lipid sera of patients with biliary cirrhosis. The clarity of the high-lipid sera in biliary cirrhosis was attributed to the hydrophilic nature of the phospholipids by virtue of their water-soluble side chains.

Whether the elevated lipo-protein observed electrophoretically as a high beta-globulin peak is merely a phenomenon of biliary regurgitation or whether it represents some derangement of hepatic metabolism remains to be determined. The patterns with high beta-globulin peaks reported by Longworth (10) and Zeldis and Alling (11) in "obstructive jaundice" tend to support the former view, but further knowledge of hepatic function in these cases would be needed. Gray and Barron (1) emphasized that jaundice *per se* has no influence on the electrophoretic pattern and presented five jaundiced cases of common duct stone, four of which exhibited normal serum proteins. The remaining case had an elevated beta-globulin fraction, but laparotomy biopsy of the liver showed marked histologic evidence of parenchymal damage. Conversely, in the present series, two cases (W. H. and M. F. with apparently quiescent pathologic processes) showed elevated beta-globulins in the presence of normal serum bilirubin.

The observations of Balfour (19) with the aid of radioactive phosphorus together with previous data (20) suggest that in patients with high levels of plasma lipids the cause of elevated phospholipids is overproduction by the liver rather than biliary regurgitation, not only in biliary cirrhosis but also in the nephrotic syndrome and other conditions.

In the present study the serial electrophoretic analyses on sera from a patient without serious

mechanical obstruction showed alteration of the serum proteins in the direction of normal coinciding with clinical improvement, as has been observed previously in patients with portal cirrhosis under medical management (21). On the other hand, as expected, two patients with irreparable stricture of the common duct showed little change in electrophoretic pattern during the period of observation.

SUMMARY

1. Electrophoretic analyses of the serum proteins of ten cases of biliary cirrhosis of the obstructive type revealed deviations from the normal composition in all.
2. The albumin fractions were diminished in all cases and the gamma-globulins were elevated in eight of the ten cases.
3. Elevated beta-globulins were found in all cases and were sometimes strikingly increased.
4. The elevation of beta-globulins was attributed to increased lipo-protein.
5. The abnormalities of the serum proteins were greatest in two cases with irreparable post-operative stricture of the common duct and secondary xanthomatosis, and were minimal in a case without clinical symptoms at the time of study.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. E. S. Guzman Barron for making available the electrophoresis apparatus used in this study, and also their indebtedness to Robert S. Levine for his invaluable technical assistance with the tests of hepatic function.

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ELECTROPHORETIC STUDIES OF THE SERUM PROTEINS IN VIRUS HEPATITIS

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The abnormal electrophoretic patterns of the serum proteins in the acute stage of virus hepatitis were first described by Gray and Barron in 1943 (1) and have since been confirmed by several investigators (2-5). In the last few years there has been considerable interest (6-13) in cases of hepatitis progressing to chronic liver disease, or developing post-necrotic cirrhosis. Except for the study by Martin (4), however, little information has been presented on the serum proteins after the acute stage of the disease.

The present investigation was undertaken to observe the alterations of the serum proteins during the course of virus hepatitis, including its later stages.

METHODS

Electrophoretic runs were performed with standard apparatus and technique, the experimental details having been given in an earlier report (14). The tests of hepatic function were performed by the usual methods as previously described (14), alkaline phosphatase being reported in Bodansky units. In our experience (15), the thymol turbidity test with buffer pH 7.55 has not infrequently given values of 4 or 5 units in normal individuals.

CASE MATERIAL

Ten volunteer subjects with normal tests of hepatic function served as controls, the data having been previously reported (14).

Twenty-one clinically typical cases of hepatitis were studied. Histologic verification of parenchymal involvement compatible with virus hepatitis was obtained in 14 cases, the tissue having been secured by one or more Vim Silverman needle biopsies or at laparotomy, and in two cases (E. H. and K. H.) at autopsy. On the basis of history the cases were considered to be infectious hepatitis with exception of two (T. H. and K. H.) regarded as homologous serum hepatitis, having received blood and plasma within 60 to 90 days prior to onset.

For the purposes of study, the material was divided into the following three groups:

Group I—Acute phase, ten patients.

Group II—Course of the disease, with serial electrophoretic studies on five patients over periods of 69 to 396 days.

Group III—Late follow-up studies, with single electrophoretic analyses on sera from 11 patients, ten to 36 months after the acute phase.

Serial needle biopsies and repeated tests of hepatic function were performed during the course of observation, as described below.

In addition to bed rest during the acute stage, the medical management included a high protein, high carbohydrate, high caloric diet containing 120 to 150 grams of protein, 350 to 450 grams of carbohydrate, a total of 3,000 to 3,500 calories, supplemented in most instances by 4 to 6 grams of choline chloride daily.

RESULTS

Group I—Acute phase

In all of the ten cases studied during the acute phase histologic examination of liver tissue was obtained, in two instances (E. H. and K. H.) at autopsy. Two of the ten cases (T. H. and K. H.) were regarded as homologous serum hepatitis, and the rest as infectious hepatitis.

Typical electrophoretic patterns are illustrated in Figure 1, and the data including tests of hepatic function are given in Table I, the cases being listed in order of increasing severity of the disease as judged by the overall clinical picture.

It will be readily observed that diminution of the albumin fraction occurred in all instances even when not evident by the Howe method. Elevation of the gamma-globulin fraction was usually but not invariably present (seven of the ten cases). G. A., J. E., and S. E. exhibited normal gamma-globulins, while H. S. had elevation of the relative proportion (*i.e.*, per cent) but not the absolute value (*i.e.*, grams per cent) of gamma-globulin. Elevated beta-globulins occurred in seven of the ten cases.

The first five cases (C. G., T. J., G. A., J. E., and S. E.) had mild hepatitis with slight to

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moderately intense jaundice. C. G. showed elevated alpha-2-, beta-, and gamma-globulins, with almost normal thymol turbidity and negative cephalin cholesterol flocculation test. T. J. exhibited elevation of all the globulins, with both thymol turbidity and cephalin cholesterol flocculation tests within normal limits. G. A., J. E. (Figure 3), and S. E. had normal gamma-globulins, the first two with elevated beta-globulins (and positive thymol turbidity and cephalin cholesterol flocculation tests), and the latter with elevated alpha-2-globulin and positive thymol turbidity test.

The last five cases (H. S., T. H., A. E., E. H., and K. H.) had severe acute hepatitis. Four of these were women after the menopause (H. S., A. E., E. H., and K. H.) and one (T. H.) was a 72 year old male with diffuse arteriosclerosis and hypertension. Three cases recovered clinically from the hepatitis and had no further symptoms

referable to liver disease (H. S., T. H., and A. E.), and two died of parenchymal liver failure with intense jaundice, edema, ascites, and hemorrhagic phenomena (E. H. and K. H.). The electrophoretic albumin values in this group were all below 3 grams per cent. H. S. (Figure 2) had only relative but not absolute increase of the beta- and gamma-globulins, while the others all showed pronounced relative and absolute gamma-globulin elevations. A. E. (Figure 4) and K. H. had elevated beta- as well as gamma-globulins. A high alpha-1-globulin occurred in T. H. The abnormalities of the serum proteins in these five cases of severe virus hepatitis were in general considerably more pronounced than in the five with clinically milder disease.

Group II—Course of the disease

The five cases with follow-up studies showed evolution of the electrophoretic patterns toward

ELECTROPHORETIC PATTERNS OF SERUM PROTEINS IN ACUTE VIRUS HEPATITIS

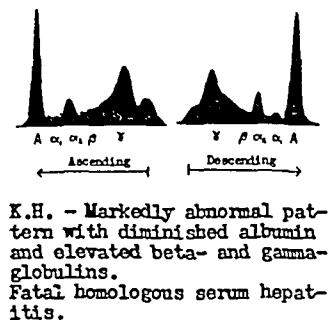
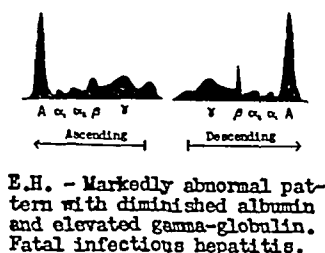
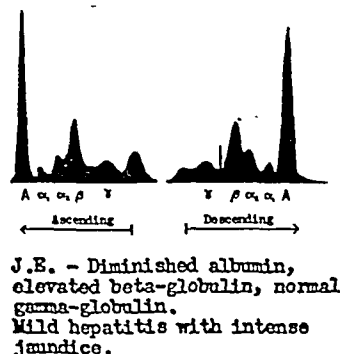
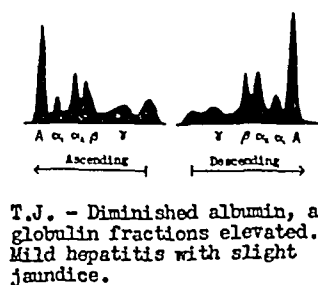
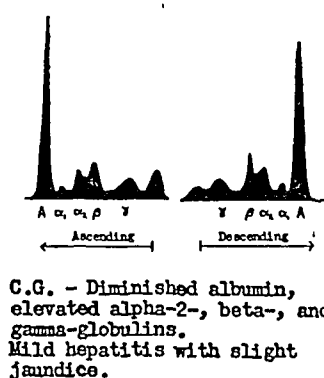
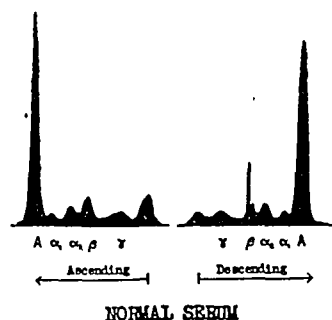


FIG. 1

The albumin peaks are indicated by "A," and the globulins by their respective Greek letter prefixes. The unlabelled peaks are the stationary anomalous boundaries due to gradients of buffer salt.

TABLE I

ACUTE VIRUS HEPATITIS

Case	Electrophoretic data											Howe sodium sulfate				Tests of hepatic function						
	per cent					grams per cent					A/G	grams per cent			bilirubin			ceph	thym	alk phos		
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ		A/G	Alb	Glob	Tot	direct	1'				15'	tot
C.G.	50.2	4.6	12.9	16.5	15.8	3.54	0.32	0.91	1.16	1.11	1.01	1.77	4.50	2.54	7.04	0.9	2.1	3.1	neg.	5.3	4.0	
T.J.	35.3	10.2	19.4	19.2	15.9	2.57	0.74	1.41	1.40	1.16	0.55	2.45	5.17	2.11	7.28	0.9	1.5	1.8	neg.	3.6	5.4	
G.A.	51.3	5.3	8.3	20.0	15.1	3.23	0.34	0.52	1.26	0.95	1.05	2.00	4.20	2.10	6.30	1.3	2.1	2.9	3+	7.4	8.2	
J.E.	43.5	5.4	9.7	29.4	12.0	2.88	0.36	0.64	1.95	0.79	0.77	1.46	3.90	2.68	6.62	7.5	11.8	15.6	3+	>20	12.1	
S.E.	46.3	7.1	20.4	11.4	14.8	3.03	0.46	1.34	0.75	0.97	0.86	1.37	3.79	2.76	6.55	3.0	4.7	7.0	2+	10.5	11.0	
H.S.	44.0	7.4	10.8	19.7	18.1	2.15	0.36	0.53	0.97	0.89	0.79	1.33	2.80	2.10	4.90	5.4	7.8	9.8	3+	7.5	9.5	
T.R.	46.0	12.3	7.3	7.3	27.1	2.55	0.69	0.41	0.41	1.50	0.85	1.19	3.02	2.54	5.56	4.6	7.6	20.8	4+	>20	17.5	
A.E.	38.4	5.3	9.8	21.7	24.9	2.80	0.39	0.71	1.58	1.82	0.62	1.39	4.25	3.05	7.30	2.4	4.7	6.7	3+	>20	11.8	
E.H.	43.5	4.4	6.8	14.0	31.3	2.08	0.21	0.32	0.67	1.49	0.77	0.96	2.33	2.44	4.77	4.0	5.8	10.4	4+	10.0	23.0	
K.H.	40.6	3.8	12.1	16.7	26.8	2.99	0.28	0.89	1.23	1.97	0.68	0.46	2.31	5.05	7.36	11.2	15.0	20.8	4+	>20	12.9	
AVERAGE	43.9	6.7	11.7	17.6	20.2	2.78	0.41	0.77	1.14	1.27	0.80	1.44	3.63	2.74	6.37							
Standard Deviation	4.7	2.7	4.5	5.7	6.3	0.43	0.16	0.35	0.43	0.39	0.15	0.52	0.92	0.83	0.93							

NORMAL SERA
(10 volunteer subjects with normal liver function tests)

AVERAGE	60.3	4.0	9.7	12.8	13.2	4.23	0.28	0.68	0.89	0.93	1.52	2.31	4.89	2.12	7.01						
Standard Deviation	2.8	0.8	1.5	1.2	1.7	0.29	0.06	0.10	0.08	0.13	0.18	0.34	0.30	0.24	0.30						

normal, but differed markedly in both the rate and extent of reversion toward normal. The changes are illustrated in Figures 2-4, and the data including tests of hepatic function are listed in Table II. The initial studies (*Day 0*) were performed during the acute phase, usually one or two weeks after the onset of the illness.

C. G., a 15 year old male, had mild acute hepatitis with slight jaundice, negative cephalin cholesterol flocculation and almost normal thymol turbidity test. A needle biopsy disclosed histologic changes compatible with virus hepatitis. The initial electrophoretic analysis (Figure 2) revealed somewhat diminished albumin despite normal Howe albumin value, and slightly elevated beta- and gamma-globulins. The jaundice subsided in two weeks; the patient became asymptomatic and remained so. The tests of hepatic function reverted to normal and the electrophoretic pattern on *Day 156* was within normal limits. A follow-up needle biopsy of the liver disclosed normal parenchyma with normal lobular architecture and minimal focal scarring.

J. E., a 35 year old female, had relatively mild

acute hepatitis, her general condition remaining quite good despite intense jaundice and pruritus. The total serum bilirubin reached a peak of 18.4 grams per cent; urobilinogen was absent from the 24 hour urine and stool collections for ten days. Microscopic findings on the needle biopsy specimen were compatible with acute virus hepatitis. The initial electrophoretic analysis (Figure 3) revealed diminished albumin and markedly elevated beta-globulin but normal gamma-globulin in the presence of a positive cephalin cholesterol flocculation test and a high thymol turbidity. Over the course of one month the jaundice and all symptoms subsided after which the patient remained asymptomatic. By *Day 20* the electrophoretic pattern had changed considerably in the direction of normal and on *Day 69*, it was within normal limits, as were the tests of hepatic function. Follow-up needle biopsy revealed marked recovery of the parenchyma with no scarring whatever.

H. S., a 72 year old female, had moderately severe acute hepatitis with intense jaundice, the serum bilirubin values attaining levels above 30

COURSE OF VIRUS HEPATITIS

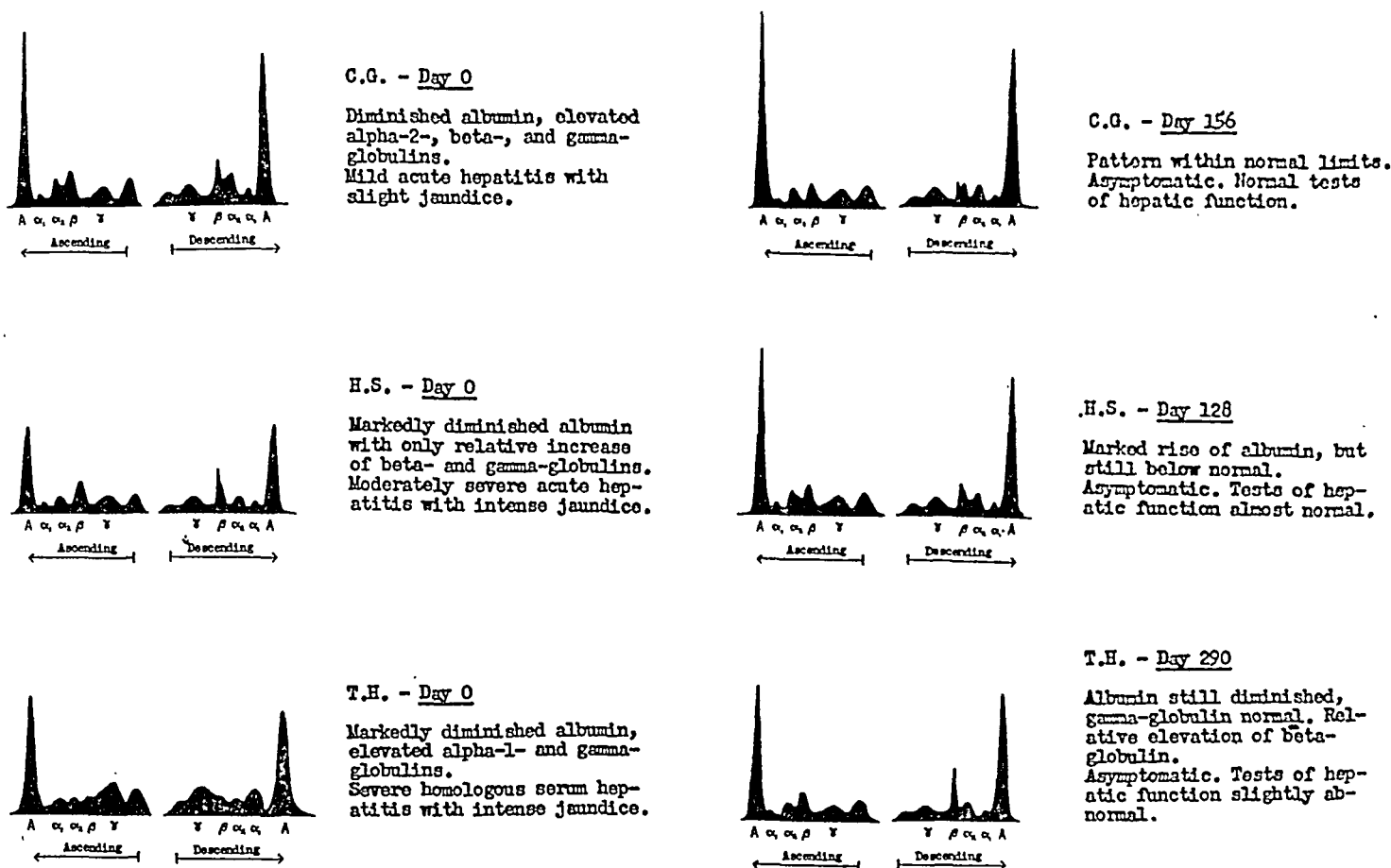


FIG. 2

mg. per cent and the tests of hepatic function revealing marked impairment. Needle biopsy disclosed acute hepatitis with some distortion of the lobular architecture. The initial electrophoretic pattern (Figure 2) was quite abnormal with very low albumin and elevation of the relative (*i.e.*, per cent) but not absolute (*i.e.*, grams per cent) values of the beta- and gamma-globulins. Within five weeks complete clinical recovery occurred with reversion of the tests of hepatic function to almost normal, and the patient remained asymptomatic. Electrophoretic analysis on *Day 128* revealed considerable change in the direction of normal with albumin rise of over 1 gram per cent, nevertheless not attaining the normal range.

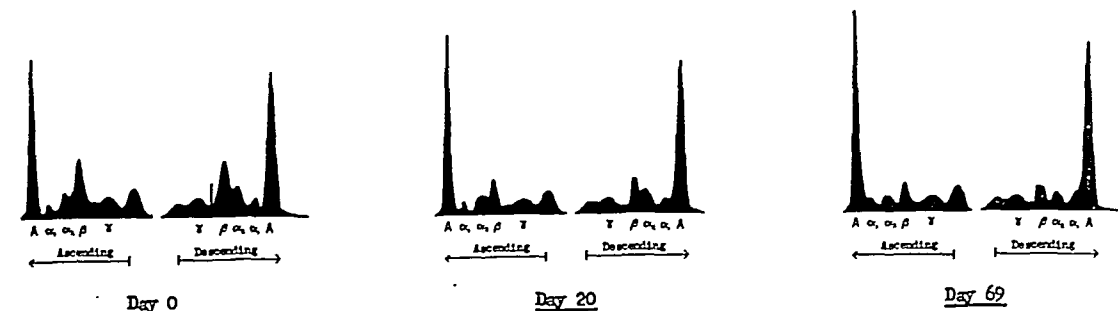
T. H., a 72 year old male, had severe acute hepatitis with intense jaundice, regarded as homologous serum hepatitis since he had received blood and plasma 90 days previously at the time of a local resection of the colon for carcinoma.

Tests of hepatic function revealed marked impairment and histologic examination of the needle biopsy specimen was compatible with virus hepatitis. The initial electrophoretic study (Figure 2) showed markedly diminished albumin and elevated alpha-1- and gamma-globulins. Complete clinical recovery occurred in approximately six weeks, the tests of hepatic function remaining slightly abnormal. Electrophoretic analysis on *Day 290* revealed some change toward normal with decline of the elevated alpha-1- and gamma-globulins, but the pattern was still definitely abnormal with diminution of albumin and relative elevation of beta-globulin.

A. E., a 67 year old female with hypertensive cardio-vascular disease but without history of cardiac failure, developed unusually severe acute hepatitis with intense jaundice, the total serum bilirubin levels exceeding 30 mg. per cent. For three weeks urobilinogen was absent from the 24

COURSE OF MILD VIRUS HEPATITIS

Sera of J.E.



Diminished albumin, markedly elevated beta-globulin, normal gamma-globulin. Mild acute hepatitis with intense jaundice.

Nearly normal pattern. Minimal jaundice.

Pattern within normal limits. Asymptomatic. Tests of hepatic function almost normal.

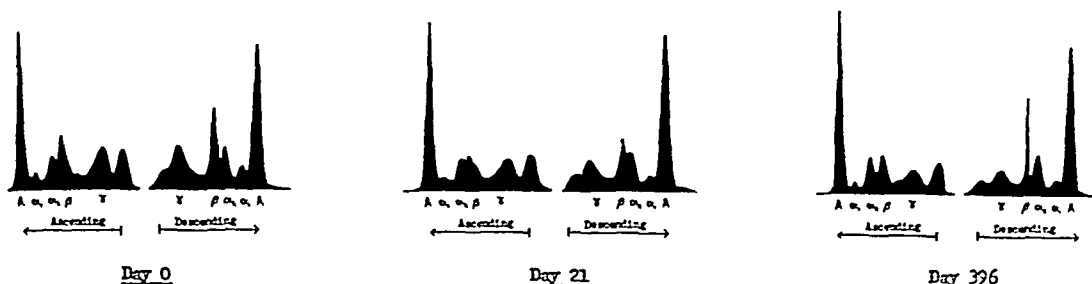
FIG. 3

hour collections of stools and urine. The other tests of hepatic function disclosed marked impairment. Needle biopsy of the liver four weeks after the onset revealed marked inflammatory infiltration with areas of liver cell necrosis and post-necrotic scarring. The initial electrophoretic pattern (Figure 4) was quite abnormal with diminished albumin and markedly elevated beta- and gamma-globulins. After eight weeks of jaundice there was gradual complete clinical re-

covery, the patient's cardio-vascular status remaining unchanged throughout. The tests of hepatic function exhibited progressive reversion to almost normal, the thymol turbidity remaining slightly elevated even after a year had elapsed. The electrophoretic pattern on *Day 21*, by which time considerable clinical improvement had occurred, was still quite abnormal but revealed a rise of the albumin and a decline of the elevated beta- and gamma-globulins. On *Day 396*, definite

COURSE OF SEVERE VIRUS HEPATITIS WITH POST-NECROTIC CIRRHOSIS

Sera of A.E.



Markedly abnormal pattern with diminished albumin, elevated beta- and gamma-globulins. Severe acute hepatitis with extreme jaundice.

Pattern still quite abnormal, but with rise of albumin, decline of beta- and gamma-globulins. Considerable clinical improvement. Minimal jaundice.

Pattern still abnormal with diminished albumin, elevated alpha-2-, beta-, and gamma-globulins. Asymptomatic. Tests of hepatic function almost normal. Post-necrotic cirrhosis histologically.

FIG. 4

TABLE II
COURSE OF VIRUS HEPATITIS

	Electrophoretic data											Howe sodium sulfate				Tests of hepatic function							
	per cent					grams per cent					A/G	A/G	grams per cent			bilirubin		ceph	thym	alk phos	ESP		
	Alb	α_1	α_2	β	γ	Alb	α_1	α_2	β	γ			Alb	Glob	Tot	direct	tot						
															1"	15"							
Sera of C.G.																							
Day 0	50.2	4.6	12.9	16.5	15.8	3.54	0.32	0.91	1.16	1.11	1.01	1.77	4.50	2.54	7.04	0.9	2.1	3.1	neg.	5.3	4.0	---	
Day 156	58.4	3.6	10.4	12.5	15.1	3.99	0.25	0.71	0.85	1.03	1.40	2.24	4.72	2.11	6.83	0.1	0.2	0.5	neg.	2.9	---	2.0	
Sera of J.E.																							
Day 0	43.5	5.4	9.7	29.4	12.0	2.88	0.36	0.64	1.95	0.79	0.77	1.46	3.90	2.68	6.62	7.5	11.8	15.6	3+	>20	18.5	---	
Day 20	55.1	5.2	11.0	14.6	14.1	3.85	0.36	0.77	1.02	0.99	1.23	1.94	4.61	2.38	6.99	1.0	1.7	2.2	3+	8.6	7.9	---	
Day 69	60.9	5.2	8.7	12.7	12.5	4.47	0.38	0.64	0.93	0.92	1.56	2.38	5.17	2.17	7.34	0.1	0.3	0.6	neg.	8.0	4.6	6.5	
Sera of H.S.																							
Day 0	44.0	7.4	10.8	19.7	18.1	2.15	0.36	0.53	0.97	0.89	0.79	1.33	2.80	2.10	4.90	5.4	7.8	9.8	3+	7.5	11.8	---	
Day 128	52.9	5.5	11.5	13.7	16.4	3.42	0.36	0.74	0.88	1.06	1.12	1.47	3.85	2.61	6.46	0.2	0.2	0.7	neg.	5.0	5.2	---	
Sera of T.H.																							
Day 0	46.0	12.3	7.3	7.3	27.1	2.55	0.69	0.41	0.41	1.50	0.85	1.19	3.02	2.54	5.56	4.6	7.6	20.0	4+	>20	15.7	---	
Day 290	52.3	4.3	9.9	18.0	15.5	2.75	0.23	0.52	0.95	0.81	1.10	1.92	3.46	1.80	5.26	0.1	0.3	1.2	neg.	5.8	2.0	11.0	
Sera of A.E.																							
Day 0	38.4	5.3	9.8	21.7	24.9	2.80	0.39	0.71	1.58	1.82	0.62	1.39	4.25	3.05	7.30	2.4	4.7	6.7	3+	>20	6.4	---	
Day 21	48.6	4.8	11.2	16.8	18.6	3.31	0.33	0.76	1.15	1.27	0.94	1.82	4.40	2.42	6.82	1.0	1.9	2.5	1+	17.2	4.5	28.0	
Day 396	46.8	4.4	14.8	15.4	18.6	3.40	0.32	1.07	1.12	1.35	0.88	2.01	4.85	2.41	7.26	0.1	0.2	0.4	neg.	9.8	6.3	6.0	

residual abnormalities were observed with diminished albumin and elevated alpha-2-, beta-, and gamma-globulins. Follow-up needle biopsy of the liver revealed definite post-necrotic cirrhosis with distortion of the lobular architecture.

Group III—Late follow-up studies

To assess the late effects of virus hepatitis, single electrophoretic analyses were performed on sera from 11 individuals who had clinically recovered from acute hepatitis during hospital admissions 10 to 36 months previously. In all cases tests of hepatic function had been carried out at intervals. None of the patients were alcoholics or had symptoms referable to the liver or gall bladder prior to the onset of acute hepatitis. Except for C. B., none had recurrent jaundice after the initial episode.

Typical electrophoretic patterns are illustrated in Figure 5 and the data including the tests of hepatic function are listed in Table III in order of increasing deviation from normal serum protein composition.

Although most of the residual abnormalities were relatively minor, only three sera (C. B.,

C. J., and W. R.) had patterns well within the normal range. The electrophoretic albumin values (absolute) were slightly diminished in four (J. G., C. P., M. K., and M. J. S.) of the 11 cases, although this was not reflected in the Howe fractionations. The gamma-globulins were elevated in six cases (G. G., R. W., J. G., C. P., M. K., and M. J. S.) and the beta-globulins in five (M. S., B. W., G. G., R. W., and M. J. S.).

Summaries of the findings in the 11 cases follow:

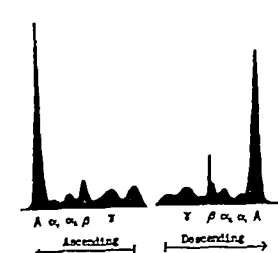
C. B., a 24 year old male, had mild infectious hepatitis with intense jaundice while in military service with recurrence of jaundice two months later. He had been asymptomatic during the ensuing 28 months. The electrophoretic pattern was within normal limits. There was definite elevation of the total serum bilirubin, other tests of hepatic function being normal.

C. J., a 29 year old male, had been asymptomatic following recovery from mild acute hepatitis 32 months previously. The electrophoretic data were in the normal range. Tests of hepatic function were normal.

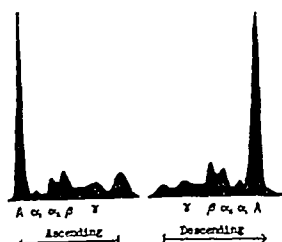
W. R., a 26 year old male, had recovered uneventfully from mild acute hepatitis 34 months previously and remained free of symptoms. The electrophoretic pattern was within normal limits. Tests of hepatic function were normal except for slightly elevated thymol turbidity (6.1 units). Needle biopsy revealed normal liver tissue.

LATE FOLLOW-UP STUDIES

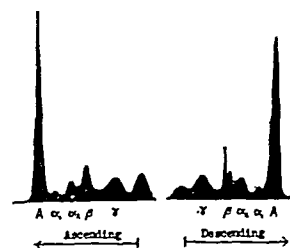
long after clinical recovery from acute virus hepatitis



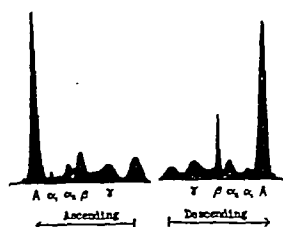
W.R. - Pattern within normal limits. Asymptomatic 3½ months after mild acute hepatitis. Almost normal tests of hepatic function. Normal liver histologically.



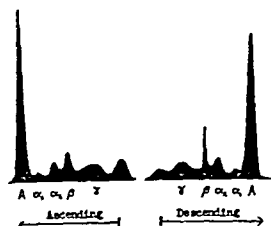
M.S. - Pattern normal except for elevated beta-globulin. Asymptomatic 1½ months after severe acute hepatitis. Normal tests of hepatic function.



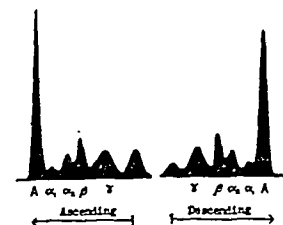
G.G. - Elevated beta- and gamma-globulins. Asymptomatic 16 months after mild acute hepatitis. Normal tests of hepatic function.



J.G. - Slightly diminished albumin, slightly elevated gamma-globulin. Asymptomatic three years after severe acute hepatitis. Almost normal tests of hepatic function. Minimal post-necrotic scarring histologically.



M.K. - Slightly diminished albumin, elevated gamma-globulin. Asymptomatic 1½ months after severe acute hepatitis. Normal tests of hepatic function. Minimal post-necrotic scarring histologically.



M.J.S. - Diminished albumin, elevated beta- and gamma-globulins. No symptoms attributable to liver disease 29 months after severe acute hepatitis. Positive cephalin flocculation and thymol turbidity tests. Post-necrotic scarring histologically.

FIG. 5

TABLE III

LATE FOLLOW-UP STUDIES

long after clinical recovery from acute virus hepatitis

Case	Months after acute stage	Electrophoretic data										Hove sodium sulfate				Tests of hepatic function								
		per cent					grams per cent					A/G	A/G	grams per cent			bilirubin		ceph	thym	alk phos	ESF %		
		Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ			Alb	Glob	Tot	direct	total						
		Alb	α ₁	α ₂	β	γ	Alb	α ₁	α ₂	β	γ	A/G	A/G	Alb	Glob	Tot	1'	15'						
C.B.	28	62.6	4.2	8.5	12.8	11.9	4.26	0.28	0.58	0.87	0.81	1.67	2.56	4.89	1.91	6.80	0.2	0.5	1.5	neg.	4.0	3.0	---	
C.J.	32	62.4	3.8	6.9	12.4	14.5	4.50	0.27	0.50	0.89	1.05	1.66	2.34	5.05	2.16	7.21	0.2	0.4	1.0	neg.	1.6	4.8	7.5	
W.R.	34	59.4	4.0	7.6	13.2	15.8	4.00	0.27	0.51	0.89	1.07	1.46	2.21	4.64	2.10	6.74	0.1	0.1	0.5	neg.	6.1	3.2	10.0	
M.S.	14	59.8	3.6	9.3	14.7	12.6	4.58	0.28	0.71	1.12	0.95	1.49	1.75	4.87	2.78	7.65	0.2	0.3	0.8	neg.	4.0	1.8	3.0	
B.W.	21	57.2	4.7	8.4	15.5	14.2	4.21	0.35	0.62	1.14	1.04	1.34	2.17	5.04	2.32	7.36	0.2	0.4	0.9	neg.	3.9	3.3	30.0	
G.G.	16	54.5	3.8	9.2	15.8	16.7	4.01	0.28	0.67	1.16	1.23	1.20	1.63	4.61	2.74	7.35	0.1	0.2	0.7	neg.	3.2	3.4	0	
R.W.	12	52.2	4.1	10.5	14.7	18.5	4.04	0.32	0.81	1.14	1.43	1.09	1.50	4.64	3.10	7.74	0	0.1	0.5	neg.	6.2	4.8	6.5	
J.G.	36	56.5	4.1	9.4	13.9	16.1	3.88	0.28	0.64	0.95	1.11	1.30	2.30	4.78	2.08	6.86	0.1	0.2	1.2	neg.	3.6	3.5	10.0	
C.P.	10	54.4	5.8	9.3	12.3	18.2	3.78	0.40	0.65	0.86	1.26	1.19	1.70	4.38	2.57	6.95	0.2	0.3	0.6	3+	8.0	4.1	27.0	
M.K.	14	55.6	3.9	10.6	12.6	17.3	3.80	0.27	0.73	0.85	1.15	1.25	1.44	4.04	2.80	6.84	0	0.2	0.7	neg.	4.5	4.0	2.5	
M.J.S.	29	48.6	4.2	10.8	15.8	20.6	3.52	0.31	0.78	1.15	1.19	0.95	1.82	4.62	2.57	7.25	0	0	0.2	4+	9.0	3.2	11.5	

M. S., a 24 year old male, had severe acute hepatitis with intense jaundice 14 months previously from which he achieved complete clinical recovery. The electrophoretic pattern was normal except for elevated beta-globulin. Tests of hepatic function were normal.

B. W., a 47 year old female, had been asymptomatic following moderately severe acute hepatitis 21 months previously. Electrophoresis disclosed elevation of the beta-globulin fraction. Bromsulfalein test showed 30 per cent retention, the other tests being normal.

G. G., a 50 year old male, had recovered from mild infectious hepatitis 16 months previously and remained asymptomatic thereafter. Electrophoretic analysis revealed elevated beta- and gamma-globulins. All tests of hepatic function were normal.

R. W., a 64 year old male, had recovered uneventfully from mild acute hepatitis 12 months previously. Electrophoresis showed elevation of the beta- and gamma-globulin fractions. The tests of hepatic function were normal except for slightly elevated thymol turbidity (6.2 units).

J. G., a 58 year old male, had been asymptomatic for 36 months following recovery from severe acute hepatitis (with laparotomy biopsy). Electrophoretic analysis revealed diminished albumin and slightly elevated gamma-globulin. The total serum bilirubin was 1.2 mg. per cent, other tests of hepatic function being normal. Two consecutive needle biopsies of the liver showed minimal post-necrotic scarring.

C. P., a 55 year old female, had recovered from moderately severe acute hepatitis ten months previously and remained free of symptoms. The electrophoretic findings were diminished albumin and elevated gamma-globulin. Bromsulfalein test showed 27 per cent retention, and the cephalin cholesterol flocculation and thymol turbidity tests were positive.

M. K., a 48 year old male, had been asymptomatic for 14 months after severe acute hepatitis. Electrophoresis revealed diminished albumin and elevated gamma-globulin. Tests of hepatic function were all normal. Needle biopsy of the liver showed minimal post-necrotic scarring.

M. J. S., a 62 year old female, had severe hepatitis (with laparotomy biopsy) 29 months previously with complete clinical recovery. There had been no symptoms referable to the liver since then. The electrophoretic pattern was the most abnormal of the group, with diminished albumin and elevated beta- and gamma-globulins. The cephalin cholesterol flocculation was 4+ and thymol turbidity was 9.0 units, the highest values in the group, the other tests of hepatic function being normal. Needle biopsy of the liver revealed post-necrotic scarring with disturbed architecture.

DISCUSSION

Electrophoretic analyses of the serum proteins in virus hepatitis were first described in 1943 by Gray and Barron (1) who reported five cases with diminished albumin in all, elevated gamma-

globulin in four, and elevated beta-globulin in two. These observations have been confirmed and extended by subsequent investigations (2-5), including the present study.

The ten cases of acute virus hepatitis studied all exhibited diminished albumin values on electrophoretic analysis, even when not evident by the Howe fractionation. Elevated gamma-globulins occurred in seven of the ten cases, and elevated beta-globulins in the same number, other variations being less frequent.

Comparison of the five clinically mild with the five severe cases of acute hepatitis revealed no strict correlation between clinical severity and extent of deviation from normal serum protein composition. This is well illustrated by the case of T. J. with quite mild clinical manifestations and slight jaundice, yet considerable abnormality of the electrophoretic pattern. On the other hand, the five severe cases all had markedly abnormal patterns, with generally greater deviations from normal than the mild cases.

The changes show similarities to those observed in a previous study of portal cirrhosis (14). The cases of portal cirrhosis with clinical manifestations such as jaundice, edema, or ascites had more pronounced abnormalities, however. In such patients, moreover, beta-globulin increases without marked elevation of gamma-globulin did not occur, in contrast to the virus hepatitis cases G. A. and J. E., with beta-globulin elevations alone. The patterns in acute virus hepatitis also resembled those observed in Weil's disease (16), chronic Brucella hepatitis (17), and infectious mononucleosis (18).

The question has been raised whether these alterations are manifestations of hepatic dysfunction or of the infectious process. Somewhat similar abnormalities have been described in infectious diseases such as syphilis, tuberculosis, typhus, malaria, lymphogranuloma venereum, leprosy, kala-azar and others (19), not believed to involve the liver, or at least not accompanied by clinical hepatic insufficiency. It must, however, be noted that most of the above conditions are characterized by chronic proliferative granulomata or by widespread cellular destruction, hence are not necessarily strictly comparable with virus hepatitis. On the other hand, in lobar pneumonia there are elevations of the alpha-2-globulins

usually without significant changes in the gamma-globulins (19, 20). Although a final answer to the problem cannot be given on the basis of the present evidence, it is suggested that the hepatic involvement in virus hepatitis plays a major role in the disturbances of the serum proteins.

The current interest in the late manifestations of virus hepatitis with prolonged hepatic dysfunction or post-necrotic cirrhosis led to the follow-up studies undertaken. To our knowledge the only previous investigation with serial electrophoretic analyses of virus hepatitis sera has been that of Martin (4) in which the maximum period of observation was 64 days. Martin's data show a number of positive findings on tests of hepatic function at the end of observation, but abnormalities of the electrophoretic patterns in only two of his 11 cases.

The present data, on the contrary, demonstrate the frequent occurrence of residual abnormalities of the serum proteins even after long asymptomatic periods.

Of the five cases followed after the acute phase, two with mild acute hepatitis showed complete reversion to normal serum protein composition. The other three with more severe acute episodes exhibited residual abnormalities of the electrophoretic pattern 128, 290, and 396 days after the initial study even though the patients were asymptomatic and had almost normal tests of hepatic function.

The late follow-up studies on sera from individuals who had clinically recovered from the acute phase of virus hepatitis ten to 36 months previously revealed frequent if usually slight abnormalities of the electrophoretic pattern. Of the 11 examined, only three patterns were considered well within the normal limits. Among the group of sera there were four instances of diminished albumin values, six elevated gamma-globulin fractions, and five elevated beta-globulins. Residual abnormalities in one or more tests of hepatic function were likewise observed. No cases of virus hepatitis with chronic jaundice were encountered.

Post-necrotic scarring was demonstrated on follow-up needle biopsy of the liver in four cases (J. G., M. K., M. J. S., and A. E.). These four patients had normal bromsulfalein tests as well as prompt (1 minute) bilirubin values (21). The

electrophoretic patterns revealed definite residual abnormalities, relatively minor in J. G. and M. K., and more marked in M. J. S. and A. E.

The occurrence of positive cephalin cholesterol flocculation and thymol turbidity tests in the presence of normal values for gamma-globulin, and conversely the occurrence of abnormalities of the serum proteins without positive flocculation or turbidity tests has been pointed out in the presentation of the data above. These findings, while of interest, are not unexpected in view of the complexity of these reactions. Recent investigations (3, 5, 22-26) have shed some light on the mechanisms of these tests, such as the evidence for the role of lipo-protein of the beta-globulin fraction in the thymol turbidity test. Nevertheless, many problems of the precise interactions of the proteins remain to be clarified.

SUMMARY

1. Electrophoretic analyses of the serum proteins in ten cases of acute virus hepatitis revealed diminished albumin values in all, elevated gamma-globulins in seven of the ten, and elevated beta-globulins in the same number.
2. Cases of severe acute hepatitis showed in general more pronounced changes than milder cases, but no strict correlation existed.
3. Serial electrophoretic analyses disclosed reversion of the serum proteins to normal in two cases of acute hepatitis, but persistent abnormalities were observed in three cases despite clinical recovery.
4. Late follow-up studies on sera from 11 individuals who had clinically recovered from acute virus hepatitis ten to 36 months previously revealed residual abnormalities in the electrophoretic patterns of eight of the 11 sera.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. E. S. Guzman Barron for making available the electrophoresis apparatus used in this study, and also their indebtedness to Robert S. Levine for his technical assistance.

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M. S., a 24 year old male, had severe acute hepatitis with intense jaundice 14 months previously from which he achieved complete clinical recovery. The electrophoretic pattern was normal except for elevated beta-globulin. Tests of hepatic function were normal.

B. W., a 47 year old female, had been asymptomatic following moderately severe acute hepatitis 21 months previously. Electrophoresis disclosed elevation of the beta-globulin fraction. Bromsulfalein test showed 30 per cent retention, the other tests being normal.

G. G., a 50 year old male, had recovered from mild infectious hepatitis 16 months previously and remained asymptomatic thereafter. Electrophoretic analysis revealed elevated beta- and gamma-globulins. All tests of hepatic function were normal.

R. W., a 64 year old male, had recovered uneventfully from mild acute hepatitis 12 months previously. Electrophoresis showed elevation of the beta- and gamma-globulin fractions. The tests of hepatic function were normal except for slightly elevated thymol turbidity (6.2 units).

J. G., a 58 year old male, had been asymptomatic for 36 months following recovery from severe acute hepatitis (with laparotomy biopsy). Electrophoretic analysis revealed diminished albumin and slightly elevated gamma-globulin. The total serum bilirubin was 1.2 mg. per cent, other tests of hepatic function being normal. Two consecutive needle biopsies of the liver showed minimal post-necrotic scarring.

C. P., a 55 year old female, had recovered from moderately severe acute hepatitis ten months previously and remained free of symptoms. The electrophoretic findings were diminished albumin and elevated gamma-globulin. Bromsulfalein test showed 27 per cent retention, and the cephalin cholesterol flocculation and thymol turbidity tests were positive.

M. K., a 48 year old male, had been asymptomatic for 14 months after severe acute hepatitis. Electrophoresis revealed diminished albumin and elevated gamma-globulin. Tests of hepatic function were all normal. Needle biopsy of the liver showed minimal post-necrotic scarring.

M. J. S., a 62 year old female, had severe hepatitis (with laparotomy biopsy) 29 months previously with complete clinical recovery. There had been no symptoms referable to the liver since then. The electrophoretic pattern was the most abnormal of the group, with diminished albumin and elevated beta- and gamma-globulins. The cephalin cholesterol flocculation was 4+ and thymol turbidity was 9.0 units, the highest values in the group, the other tests of hepatic function being normal. Needle biopsy of the liver revealed post-necrotic scarring with disturbed architecture.

DISCUSSION

Electrophoretic analyses of the serum proteins in virus hepatitis were first described in 1943 by Gray and Barron (1) who reported five cases with diminished albumin in all, elevated gamma-

globulin in four, and elevated beta-globulin in two. These observations have been confirmed and extended by subsequent investigations (2-5), including the present study.

The ten cases of acute virus hepatitis studied all exhibited diminished albumin values on electrophoretic analysis, even when not evident by the Howe fractionation. Elevated gamma-globulins occurred in seven of the ten cases, and elevated beta-globulins in the same number, other variations being less frequent.

Comparison of the five clinically mild with the five severe cases of acute hepatitis revealed no strict correlation between clinical severity and extent of deviation from normal serum protein composition. This is well illustrated by the case of T. J. with quite mild clinical manifestations and slight jaundice, yet considerable abnormality of the electrophoretic pattern. On the other hand, the five severe cases all had markedly abnormal patterns, with generally greater deviations from normal than the mild cases.

The changes show similarities to those observed in a previous study of portal cirrhosis (14). The cases of portal cirrhosis with clinical manifestations such as jaundice, edema, or ascites had more pronounced abnormalities, however. In such patients, moreover, beta-globulin increases without marked elevation of gamma-globulin did not occur, in contrast to the virus hepatitis cases G. A. and J. E., with beta-globulin elevations alone. The patterns in acute virus hepatitis also resembled those observed in Weil's disease (16), chronic Brucella hepatitis (17), and infectious mononucleosis (18).

The question has been raised whether these alterations are manifestations of hepatic dysfunction or of the infectious process. Somewhat similar abnormalities have been described in infectious diseases such as syphilis, tuberculosis, typhus, malaria, lymphogranuloma venereum, leprosy, kala-azar and others (19), not believed to involve the liver, or at least not accompanied by clinical hepatic insufficiency. It must, however, be noted that most of the above conditions are characterized by chronic proliferative granulomata or by widespread cellular destruction, hence are not necessarily strictly comparable with virus hepatitis. On the other hand, in lobar pneumonia there are elevations of the alpha-2-globulins

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The late follow-up studies on sera from individuals who had clinically recovered from the acute phase of virus hepatitis ten to 36 months previously revealed frequent if usually slight abnormalities of the electrophoretic pattern. Of the 11 examined, only three patterns were considered well within the normal limits. Among the group of sera there were four instances of diminished albumin values, six elevated gamma-globulin fractions, and five elevated beta-globulins. Residual abnormalities in one or more tests of hepatic function were likewise observed. No cases of virus hepatitis with chronic jaundice were encountered.

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INFLUENCE OF THE ADRENAL CORTEX ON BODY WATER DISTRIBUTION AND RENAL FUNCTION¹

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A relation between the adrenal cortex and the distribution of body water has been recognized since the first demonstration that adrenal insufficiency was associated with a marked degree of hemoconcentration (1, 2). The suggestion that this hemoconcentration might be attributed to a decrease in plasma volume (3, 4) was later confirmed by direct measurement (4-8), and by the circumstance that normal plasma volume was restored with adequate desoxycorticosterone therapy (6-9).

The administration of cortical extract to animals in adrenal insufficiency was followed by hemodilution and diuresis even though no water was ingested (10, 11). Swingle and his collaborators suggested that adrenalectomized animals were unable to mobilize fluids from the interstitial and cell space (10), and that the hormone effected the redistribution of fluids in the organism (12). According to Harrop (11), fluid entered cells during insufficiency and was released by therapy. The extracellular space, as judged by the volume of distribution of chloride and thiocyanate, was decreased after adrenalectomy and restored by hormone therapy (7, 8, 11, 13). Moreover, the picture of adrenal insufficiency was simulated by peritoneal dialysis, a procedure which reduces extracellular fluid volume (11, 14, 15, 16).

As a consequence of the changes noted in serum electrolyte pattern during adrenal insufficiency (1, 2, 17), attention was focused on the simultaneous electrolyte and water changes in the tissues (16, 18-23). Calculations of the tissue water compartments, based upon the assumption that chloride is limited to extracellular distribution, revealed a

shift of water from the interstitial into the intracellular space (20-23).

Marshall and Davis (24), by measuring the excretion of phenolsulfonephthalein, urea and creatinine, demonstrated a decrease in renal function following complete adrenalectomy. The subsequent use of more precise measures of renal activity confirmed the latter observation. Inulin, creatinine, urea and diodrast clearances as well as diodrast T_m were found to be reduced after adrenalectomy and restored after adequate hormone therapy (25-29).

In view of the above evidence, it seemed desirable to measure changes in all the body water compartments and to correlate them with modifications in renal function observed simultaneously in animals subject to alterations of adrenal function. Previous methods for the estimation of the extracellular space used either thiocyanate or chloride, both of which substances are now known to enter cells in a variable proportion (30-36). To overcome this objection a more accurate method (inulin space) for the determination of the extracellular volume was devised (37, 38). Coupled with the use of heavy water (D_2O) as a measure of the total body water, accurate estimates of intracellular volume were feasible. The use of radioactive sodium (Na^{24}) or potassium (K^{42}) afforded means for determining the intracellular distribution of these ions.

METHODS

Extracellular space was measured as the volume of distribution of inulin. The total inulin recovered, after the cessation of a constant intravenous equilibrating infusion, corrected for urinary delay time, and divided by the plasma concentration at the moment of the interruption of the infusion, equals the volume of distribution of inulin (38).

Total body water was determined by the space of distribution of heavy water (D_2O). A known amount of D_2O was injected intravenously and blood samples were drawn two and three hours later. The bloods were

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analyzed for D₂O concentration by the falling drop method of Keston, Rittenberg and Schoenheimer (39). Intracellular volume was calculated as the difference between the D₂O and the inulin spaces. The plasma volume was determined with T-1824 (40) and the thiocyanate space was measured by the procedure of Crandall and Anderson (30).

Inulin clearance (C_{In}), p-aminohippuric acid clearance (C_{PAH}) and maximal tubular excretory capacity for p-aminohippurate (T_{mPAH}) were determined during the equilibrating inulin infusion following the standard procedure of Smith and his co-workers (41). The chemical analyses were done with the method of Harrison (42) for inulin and of Bratton and Marshall (43) modified (41) for PAH.

Plasma sodium and potassium were measured with an internally compensated Perkin-Elmer flame photometer. The total cation in the body was determined by "in vivo" dilution of Na²⁴ or K⁴², the radioactivity of the plasma being measured with a Geiger-Müller counter on a three-hour sample for Na²⁴ and on a nine-hour sample for K⁴² (44, 45). The average intracellular cation concentration was calculated according to a method described previously (44, 45). The plasma NPN was determined by the Folin and Wu method (46), and plasma specific gravity with the copper sulphate method (47). The blood pressure was measured directly in the femoral artery (Tycos gauge) and the hematocrit determined by the usual procedure.

Observations were made on normal, trained, unanes-

TABLE I
Effect of DCA in normal dogs

Dog	Experiment	Day	Volumes of distribution									Renal function				Plasma concentration	Blood pressure
			Inulin	D ₂ O	Intra-cellular water	SCN	T-1824	Fraction of body weight			C_{In}	C_{PAH}	Filtration fraction	T_{mPAH}	K		
								Inulin	D ₂ O	Intra-cellular							
1	Average of three controls		cc.	cc.	cc.	cc.	cc.	cc./kg.	cc./kg.	cc./kg.	cc./min.	cc./min.	per cent	mg./min.	mEq./liter	mm. of Hg	
			3,010	10,300	7,290	4,650	1,020	201.	687.	486.	73.4	216	34.0	14.6	4.0	110	
	Treatment with 20 mg. DCA daily for 16 days	4	3,690			4,760	1,046	246.			76.7	210	36.5	9.5		110	
	40 mg. daily for six additional days	10	4,820	9,990	5,170	5,510	976	307.	634.	327.	100.0	284	35.2	10.6	3.0	104	
	After treatment*	16	3,270	8,270	5,000	4,700	1,070	243.	615.	372.	80.1	200	40.0	12.0	3.0	105	
		23	2,940	9,750	6,810	5,000	1,046	211.	701.	490.	65.7	217	30.3	11.1	2.9	102	
		30	2,855	10,370	7,515	4,535	1,054	193.	701.	508.	66.2	220	30.0	16.8	4.2	102	
2	Average of three controls		3,000	11,150	8,150	5,250	1,160	176.	656.	480.	88.0	320	27.5	23.0	3.8	113	
	Treatment with 30 mg. DCA daily	3	3,380			6,090	1,000	203.			77.3	240	32.2		3.9	114	
		6	3,865	10,900	7,035		1,420	223.	628.	405.	95.0	365	26.0	22.6	2.7	116	
		9	4,190	11,500	7,310	6,170	1,150	257.	706.	449.	97.5	314	31.0	5.8	2.5	116	
		13	4,360	11,100	6,740	7,610	1,250	252.	642.	390.	123.0	394	31.2	17.5	2.2	115	
		16	4,540	10,350	5,810		1,530	246.	562.	316.	116.3	344	33.8	13.4	2.1	108	
	After treatment*	21	4,000	10,680	6,680	5,690	1,400	230.	614.	384.	116.5	433	27.0	28.0	2.4	110	
		13	3,830	11,300	7,470	6,950	1,340	225.	665.	440.	100.0	308	32.5	24.4	4.2	118	
3	Average of three controls		3,650	12,400	8,750	6,380	1,180	190.	645.	455.	60.1	180	33.4	19.3	3.9		
	Treatment with 30 mg. DCA daily	2	4,170	10,900	6,730	6,920	1,024	222.	579.	357.	71.0	217	32.7	13.0	3.6		
		5	4,920	11,840	6,920	6,870	1,185	256.	616.	360.	73.0	242	30.2	18.0	2.5		
		11	5,540	11,000	5,460	7,300	1,260	267.	527.	260.	92.2	256	36.0	15.0	2.0		
		17	4,730	12,900	8,170	7,010	992	231.	631.	400.	80.0	257	31.1	16.8	2.8		
		24	3,920	12,400	8,480	6,560	1,340	192.	608.	416.	79.5	231	34.4	18.8	2.8		

* "After treatment" represents the number of days which have elapsed since therapy was discontinued.

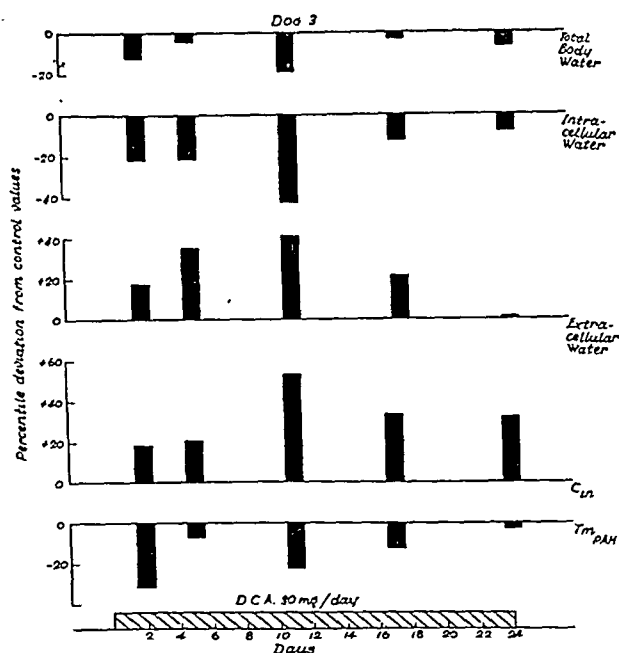


FIG. 1. ACTION OF DCA IN A NORMAL DOG

The results of Dog 3 reported in Table I are expressed as percentile deviations from the control values in relation to time.

thetized female dogs in a 24-hour fasting state, and an identical procedure for clearances and space determinations was followed in each experiment. At least three complete control experiments of this type were performed, and then the animals were divided into three groups: one was treated with desoxycorticosterone acetate (DCA),⁴ the second with adrenal cortical extract⁵ and the third was adrenalectomized and maintained exclusively on DCA, studies being made before and after withdrawal of DCA. In the first two groups, the dogs were followed until the physiological variables had returned to control values.

RESULTS

Action of desoxycorticosterone acetate: Three normal dogs were injected intramuscularly with 20 or 30 mg. of desoxycorticosterone acetate (DCA) in oil daily for three weeks, four to six series of observations being made during that period (Table I, Figure 1). The most pronounced and earliest response noted was a change in the distribution of body water. Whereas the total body water remained nearly constant, falling about

10 per cent from the control value, the intracellular volume progressively decreased while the extracellular volume expanded. This fluid shift was apparent on the second day of treatment (Dog 3, Figure 1) and attained its maximum on the 10th or 11th day when the intracellular volume in Dogs 1, 2 and 3 diminished 33, 34 and 43 per cent, respectively, and the extracellular space increased 53, 46 and 40 per cent.

The volume of distribution of thiocyanate qualitatively tended to follow the extracellular space, as measured by inulin, but no constant relation was maintained between them. Plasma volume in general did not change significantly, despite the large transfers of water in the other compartments; Dog 2, however, showed an irregular increment of about 30 per cent from control values with no definite relation to the changes in extracellular fluid.

At the same time that the redistribution of body water occurred, there was a progressive increase in filtration rate of 37, 40 and 53 per cent in Dogs 1, 2, and 3, respectively, an increase which was fairly well correlated with the expansion of the extracellular space, the peak changes coinciding in time. The renal plasma flow also increased, but to a lesser extent, with a consequent increase in the filtration fraction. Tm_{PAH} , on the contrary, was reduced to 26, 74 and 32 per cent, without any obvious correlation with the other changes.

The serum potassium fell to a little above half of its control value, but no changes were detected in plasma sodium. Plasma specific gravity and the hematocrit both declined slightly during the

TABLE II
Dog 1

Experiment	Day	Total Na	Intra-cellular Na	Intra-cellular water	Average intracellular Na concentration
Control		mEq. 606	mEq. 170	cc. 7,290	mEq./liter 22
Treatment with 20 mg. DCA daily for 16 days	16	785	284	5,000	56.8
40 mg. daily for the following six days	23	750	304	6,810	44.7
After treatment*	30	642	210	7,515	28.0

* "After treatment" represents the number of days which have elapsed since therapy was discontinued.

⁴ The desoxycorticosterone acetate used was supplied by Ciba Pharmaceutical Products, Summit, New Jersey, and by Roche-Organon Inc., Nutley, New Jersey.

⁵ Part of the Upjohn's adrenal cortical extract utilized was supplied by the Upjohn Company, Kalamazoo, Michigan.

TABLE III
Dog 3

Experiment	Day	Total K	Intra-cellular K	Intra-cellular water	Average intracellular K concentration
		mEq.	mEq.	cc.	mEq./liter
Control		962	948	8,750	108
Treatment with 30 mg. DCA daily	11	845	834	5,460	152

period of treatment, while no significant variations were observed in the mean blood pressure or in the plasma non-protein nitrogen concentration.

In each of the dogs the modifications noticed as a consequence of the treatment augmented progressively, reaching a maximum approximately on the 11th day, and then tended to return to normal despite the continuation of the injection of DCA and despite an increase in dosage in Dog 1. The

plasma potassium concentration was the sole exception in that it reverted only partially after having reached its lowest level. Two to six weeks after the treatment had been discontinued, all the animals had returned to normal.

The changes in total body sodium were studied in Dog 1 (Table II). During treatment with DCA the total body sodium increased by 30 per cent over the control value, with a simultaneous increase in the average intracellular concentration of this ion.

Total potassium was measured in Dog 3 (Table III) and at the height of the response to the treatment, when the serum potassium was 2 mEq./liter, it had decreased 12 per cent; since the intracellular water had decreased by 39 per cent, intracellular concentration had increased significantly.

Action of adrenal cortical extract: Three normal dogs (Dogs 4, 5 and 6) were treated daily

TABLE IV
Effect of total adrenal cortical extract in normal dogs

Dog	Experiment	Day	Volumes of distribution								Renal function				Plasma	Blood pressure
			Inulin	D ₂ O	Intra-cellular water	SCN	T-1824	Fraction of body weight			C _{In}	C _{PAH}	Filtration fraction	T _{mPAH}	K	
								Inulin	D ₂ O	Intra-cellular						
4	Average of three controls		cc. 2,450	cc. 6,830	cc. 4,380	cc. 4,510	cc. 960	cc./kg. 201.	cc./kg. 560.	cc./kg. 359.	cc./min. 67.7	cc./min. 192	per cent 35.2	mg./min. 11.8	mEq./liter 3.8	mm. of Hg 118
	Treatment with 15 cc. of extract daily	5	2,620	9,110	6,490	4,790	1,052	201.	700.	500.	66.4	248	26.8	20.5	3.8	114
	After treatment*	12	2,870	8,620	5,750		1,046	221.	663.	442.	75.2	225	33.4	14.0	4.0	125
		35	2,510	7,100	4,590	4,700	917	200.	568.	368.	58.0	160	36.2	11.3	4.0	120
5	Average of three controls		2,050	6,380	4,330	3,500	580	195.	608.	412.	45.5	170	26.8	7.4	4.0	
	Treatment with 15 cc. of extract daily	3	1,980	8,300	6,320	3,900	710	180.	755.	575.	46.3	124	37.3	6.6	4.4	
		6	2,030	7,360	5,330	3,780	580	207.	750.	543.	47.7	161	29.7	7.0	4.0	
	After treatment*	11	1,960	6,860	4,900	3,100	598	190.	663.	473.	43.3	116	37.3	10.9	4.1	
		8	2,160	6,200	4,040	3,390	538	217.	624.	407.	52.4	146	35.9	5.6	3.7	
6	Average of two controls		2,800	8,000	5,200	5,590	830	215.	615.	400.	109.0	300	36.3	11.0	4.1	
	Treatment with 15 cc. of extract daily	4	3,560	8,700	5,140	5,160	890	263.	644.	381.	105.0	316	33.2	5.0	3.1	
		10	3,890	11,100	7,210	7,210	1,030	264.	763.	499.	89.0	233	38.1	14.0	3.0	

* "After treatment" represents the number of days which have elapsed since therapy was discontinued.

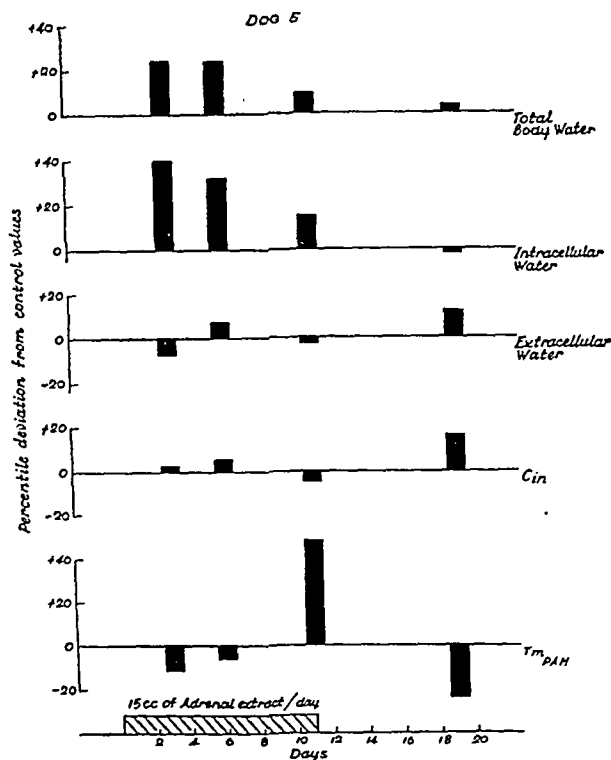


FIG. 2. ACTION OF THE ADRENAL CORTICAL EXTRACT IN A NORMAL DOG

The results of Dog 5 taken from Table IV are expressed as percentile deviations from the control values in relation to time.

with 15 cc. of Upjohn's beef adrenal cortical extract for 13 days, and two or three observations were made during that period (Table IV, Figure 2).

The changes obtained differed markedly from those produced by DCA and involved body water distribution without alterations in renal function or plasma electrolyte levels. Cortical extract caused an increase in the intracellular fluid volume and in total body water, with little or no change in the extracellular space. The enlargement of the intracellular space amounted to 40, 39 and 25 per cent of control values and was apparent on the third day of the treatment (Figure 2). The extracellular space did not change in Dogs 4 and 5 and increased moderately (23 per cent) in Dog 6. Thiocyanate volume followed these variations in a somewhat irregular manner. Plasma volume did not show any significant change.

There was no marked change in C_{in} or C_{PAH} , although the former again showed a fair correlation with the extracellular volume. Tm_{PAH} in-

creased almost 74 per cent in Dog 4 and in the other two animals first decreased and subsequently rose above control values.

Plasma sodium, potassium and non-protein nitrogen concentration did not vary to any significant extent, nor did the hematocrit or the mean blood pressure. No determinations of the total cation in the body were made.

The modifications in the intracellular space induced by cortical extract also tended to disappear under prolonged treatment.

Effect of total adrenalectomy: Two normal dogs (Figures 3 and 4, and Table V) were completely adrenalectomized in two stages and maintained with 10 and 15 mg. of DCA daily (Dogs 7 and 8).⁶ While still under treatment and showing no signs of adrenal insufficiency, an experiment was performed six days after complete adrenalectomy in Dog 8 and after 21 days in Dog 7. In both animals the changes from preoperative values were similar to those observed in normal dogs treated with DCA, i.e., intracellular water was reduced

⁶ The dogs were maintained on their usual diet. No sodium chloride was added.

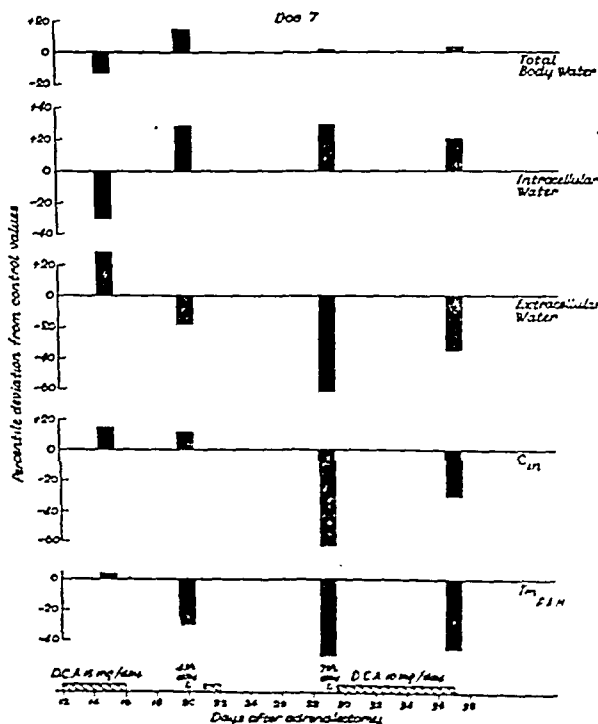


FIG. 3. EFFECT OF TOTAL ADRENALECTOMY ON DOG 7

The results of Table V are expressed as percentile deviations from the control values in relation to time.

TABLE V
Effect of adrenalectomy in normal dogs

Dog	Experiment	Day	Volumes of distribution								Renal function				Plasma concentration			Blood pressure
			Inulin	D ₂ O	Intra-cellular water	SCN	T-1824	Fraction of body weight			C _{In}	C _{PAH}	Filtration fraction	T _{mpAH}	K	Na	mg. per cent	
								Inulin	D ₂ O	Intra-cellular								
7	Average of two controls Adrenalectomized, treated with 15 mg. DCA daily Treatment interrupted Treatment resumed 10 mg. DCA daily		cc. 2,600	cc. 8,400	cc. 5,800	cc. 4,320	cc. 950	cc./kg. 196	cc./kg. 632	cc./kg. 436	cc./min. 71.5	cc./min. 227	per cent 31.5	mg./min. 16.2	mEq./liter 4.5	mEq./liter 152	mg. per cent 18.0	mm. of Hg 125
		21	3,740	8,350	4,610	5,700	890	249	556	307	81.9	255	32.1	16.6	3.0	153	20.1	130
		4	2,450	10,900	8,450	6,840	856	162	722	560	80.0	238	33.8	11.6	6.0	141	45.0	102
		7	970	8,100	7,130	4,070	604	76	637	561	26.8	110	24.4	8.3	5.0	153	70.6	64
		8	1,610	8,100	6,490	5,400	710	131	659	528	50.9	196	26.0	9.1	3.2	141	16.0	97

8 Average of